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Studies On The Hemoglobin Alpha -subunit And Its Interaction With Haptoglobin

Frank Alexander Terpstra

ACKNOWLEDGEMENTS

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Special thanks are due Mr. Walter P. Chung for his superb technical assistance. The author greatly admires Walter and feels his contribution to our laboratory is invaluable.

STUDIES ON THE HEMOGLOBIN α-SUBUNIT AND ITS INTERACTION WITH HAPTOGLOBIN

by

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ABSTRACT

Although it is accepted that the α subunit of hemoglobin binds to haptoglobin, the stoichiometry of this interaction has been the subject of debate. Much of the work in this area has been marred by α subunit instability and inadequate proof of purity. These shortcomings are here avoided and the Hp:α interaction has been further characterized.

Homogeneous porcine haptoglobin was isolated in gram quantities by extending published methods to include preparative gel electrophoresis as well as affinity chromatography involving Concanavalin A- Sepharose 4B. The latter was found to be most suitable for large scale purification but necessitated the use of Hp material prepurified of other glycoproteins. The presence of dimer albumin which complicated other modes of separation such as gel filtration was found to be irrelevant as might be expected.

Human hemoglobin α subunits were prepared by the

method of Geraci, Parkhurst and Gibson (1969) with modification. EDTA was included in all solutions employed. Conditions of 0.1 M phosphate pH 7.5 - 2×10^{-5} M EDTA were conducive to subunit stability. In addition, G-75 Sephadex chromatography was necessary to remove a slight hemoglobin contamination. The resulting subunits were native and pure by several criteria.

As a preliminary to Hp binding studies, the extinction value of α^{SH} was determined at 368 nm as was its stability to acidic and basic conditions.

Attempts made to isolate complex formed by Hp and α^{SH} were fruitless and considered impractical. Plateau height analysis of (Hp + α) sedimentation behavior indicated the presence of an equilibrium with maximum binding of two subunits per Hp at a six fold α:Hp molar ratio. This was supported by comparison of various sedimentation coefficients and confirmed by supra plateau gel filtration studies.

Self-association of α^{SH} to form dimer was studied by a plateau edge technique as well as by sedimentation equilibrium ultracentrifugation. The dissociation constant and ΔG_d^o were calculated in a moderate and high ionic strength environment.

Crosslinking α^{SH} with the bifunctional imidoester dimethyl adipimidate in conditions which yield intramolecular bridges resulted in two electrophoretically separable dimers and uncrosslinked DMA-α monomer. The

ability of these components as well as ethyl acetimidate-reacted α^{SH} to form complex with haptoglobin was investigated.

The following theory was proposed: α subunits spontaneously form a dimer analogous to hemoglobin $\alpha_1\beta_1$ and experience a conformational transition which makes them complementary to haptoglobin. When this dimer is artificially maintained by DMA, significant binding affinity for Hp results. It was also suggested that this binding involves an α- and β-specific site on the Hp surface and that both exist in free Hp. Two DMA-α dimers were predicted to form complex with Hp. This theory was expanded to apply to the formation of HpHb complex. Specifically, isolated α and β subunits undergo a conformational rearrangement upon association as an $\alpha_1\beta_1$ dimer which makes them complementary to Hp.

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To those I love and to those who love me.

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ABBREVIATIONS

βME	β-mercaptoethanol
CM	Carboxymethyl
DEAE	Diethylaminoethyl
DMA	Dimethyl Adipimidate
EDTA	Ethylene diamine tetraacetic acid
GLU	α-D-glucose
GuHCl	Guanidine Hydrochloride
PCMB	p-chloromercuribenzoate
PMB	p-mercuribenzoate
SDS	Sodium dodecyl sulfate
TRIS	2-amino-2-hydroxymethylpropane-1,3-diol
PAGE	Polyacrylamide gel electrophoresis
TLG	Thin Layer gel filtration
-SH	Sulfhydryl group
-S-S-	Disulfide bridge
α^{SH}	Native ferrous hemoglobin α subunit. Also denoted α.
α^{PMB}	α subunit with sulfhydryl modified by PCMB
β^{SH}	Native ferrous hemoglobin β subunit. Also denoted β.
β^{PMB}	β subunit with one or both sulfhydryls modified by PCMB
Hb	Hemoglobin. Also denoted $(\alpha\beta)_2$
Hp	Haptoglobin
HpHb	Haptoglobin hemoglobin complex
$Hp\alpha_n$	Complex of haptoglobin and n α subunits
Con A	Concanavalin A

INTRODUCTION

Haptoglobin is an α_2 plasma protein for which several physiological roles have been proposed. The ability of Hp to form a 1:1 stoichiometric complex with Hb and thus prevent glomerular filtration of extracorpuscular hemoglobin is most striking. Due to the remarkable stability of the HpHb complex and commonality with antigen-antibody systems, the study of its nature has drawn considerable attention of protein chemists. With continual advancement in protein purification and analysis methodology has come an increasing body of information.

Hemoglobin, the tetrameric respiratory chromoprotein of the erythrocyte, is undoubtably the most intimately understood allosteric protein. Dissociation of this tetramer to $\alpha\beta$ dimers has been shown to precede interaction with haptoglobin (Nagel and Gibson, 1971). On the basis of interaction studies of Hp with isolated Hb subunits, it has been noted that the α subunit binds with an as yet unquantified affinity to Hp. An equilibrium is most probably involved but conflicting estimates of the maximum

number which bind have appeared in the literature. The major immediate goal of this work is the resolution of this uncertainty. Toward this end, chromatographic separation of the complex will be undertaken. If the $Hp\alpha_n$ complex, in contrast to HpHb, is found to be insufficiently stable to isolation, analysis methods of complex composition in an α environment will be required.

A prerequisite to these studies is the preparation of pure and native Hp and α. The former has been purified to homogeneity but only by procedures which conveniently yield milligram quantities. It is hoped that these procedures can be extended to eliminate this deficiency. The latter have been prepared by several variations of the basic method of Bucci and Fronticelli (1965) involving forced dissociation upon reaction with p-chloromercuri-benzoate. The modification of Geraci, Parkhurst and Gibson (1969) seems most suited for this task.

Little is known about the physicochemical properties of the isolated α subunit, specifically self-association behavior. Subsequent to the establishment of α subunit quality, the opportunity to investigate this subject may be pursued.

The ultimate aim of this work is to gain some insight into the Hp-Hb system. The interaction of α with Hp affords a more simple and perturbable system with which to approach the larger topic.

REVIEW OF THE LITERATURE

HEMOGLOBIN

Structure

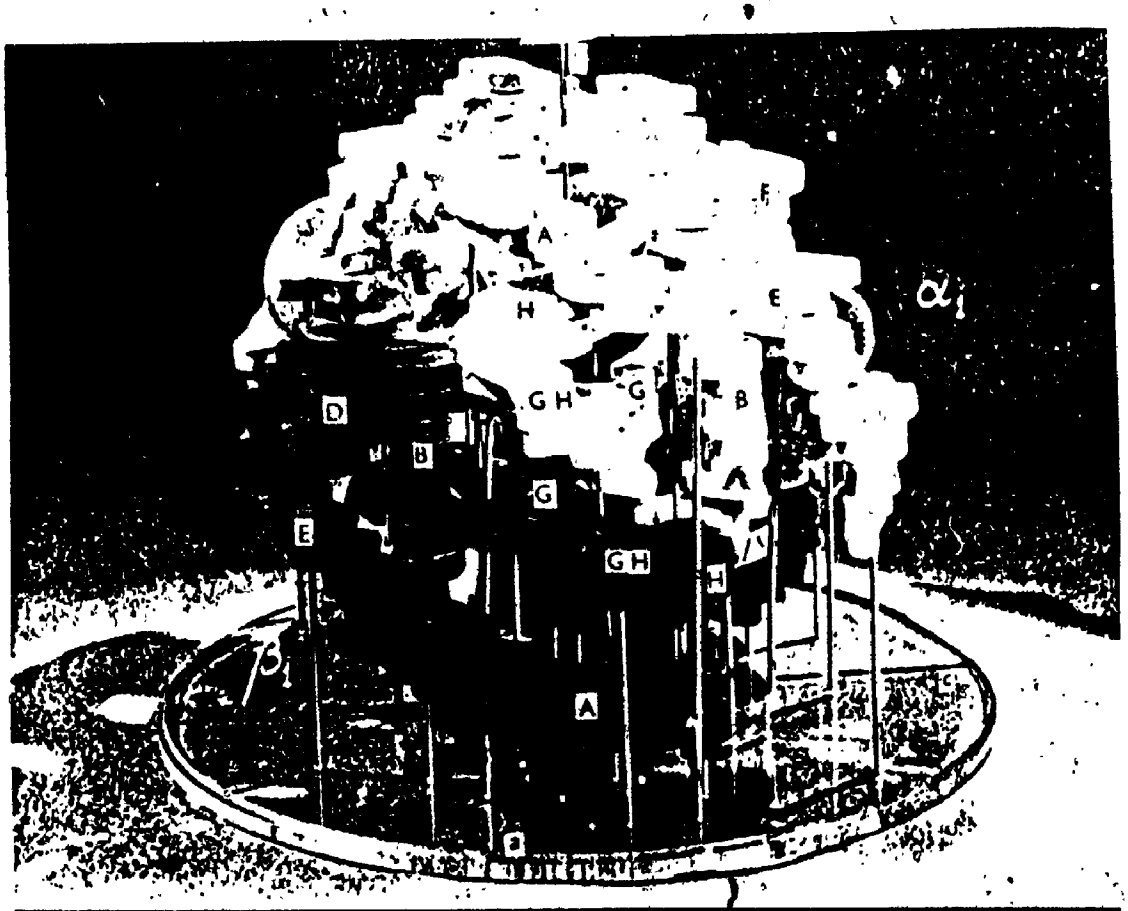
Hemoglobin, the respiratory chromoprotein of red blood cells transports molecular oxyden from the lungs through the arterial system to the peripheral tissues and is also intimately involved in the return transport of carbon dioxide. More is known about the primary to quaternary structure of the four constituent subunits, the forces that bind them together and the allosteric mechanisms that make hemoglobin so eminently suitable for its role, than any other oligomeric protein.

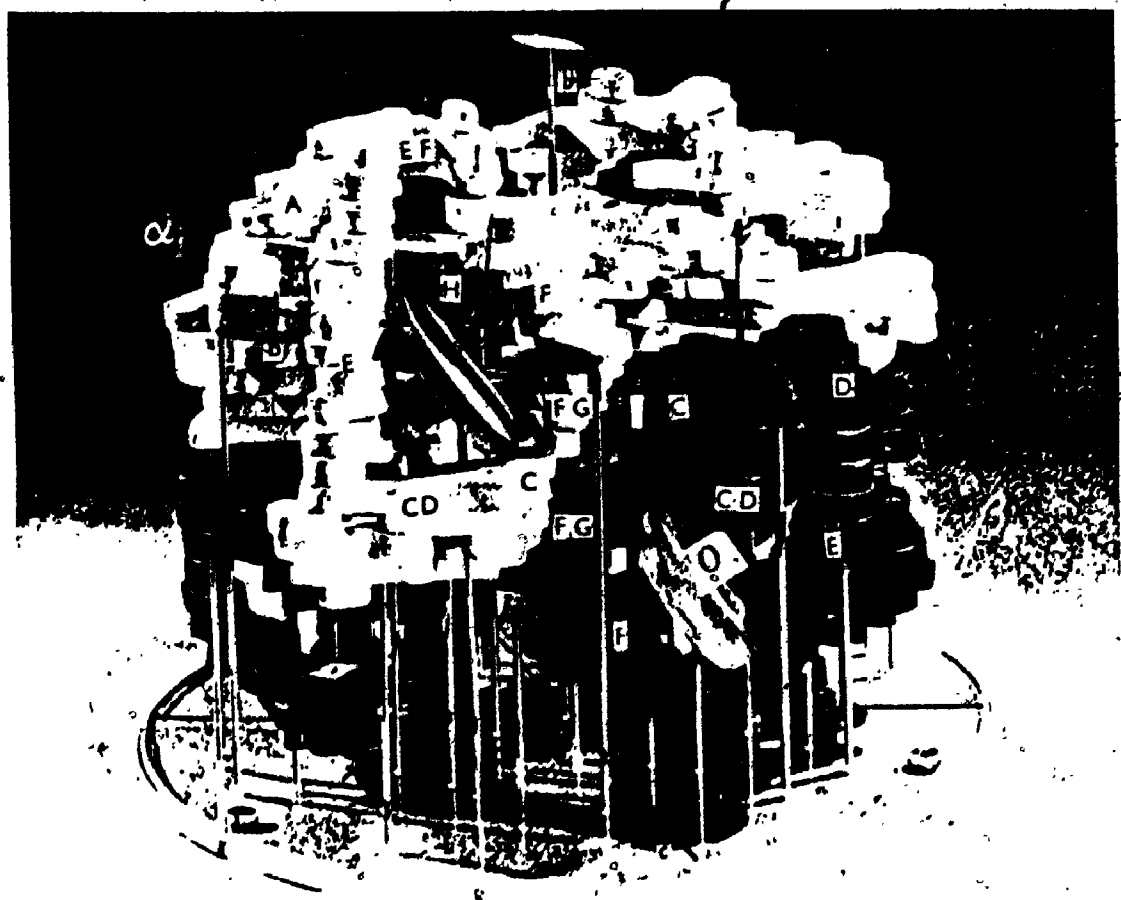
The physiological form of the molecule is a tetramic dimer of dimers consisting of two pairs of similar polypeptides, α and β subunits, arranged tetrahedrally resulting in two axes of symmetry. Contact areas between the two sets of subunits, denoted by the subscripts 1 and 2, are not equivalent. In Figure 1, a model of horse hemoglobin at 5.5 Å resolution illustrates the two interfaces between the two αβ dimers

Figure 1: Model of horse hemoglobin at 5.5 Å resolution (Cullis et al, 1962; Perutz, 1969).

Above: View along the axis to show the $\alpha_1\beta_1$ contact.

Below: View perpendicular to that axis to show the $\alpha_1\beta_2$ contact.


A
H
E
α_1
GH
G
B
D
B
G
E
GH
A


α_1
A
H
F
F
FG
C
D
CD
C
CD
FG
E
F

commonly referred to as the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ contact areas, as well as the two axes of symmetry (Cullis et al, 1962; Perutz, 1969). The dimers are positioned so as to create an open-ended internal cavity populated by a variety of polar side chains and filled with water. This cavity allows spatial changes upon the binding or release of oxygen or other ligand.

The individual α and β subunits consist of 141 and 146 amino acids respectively most of which participate in helically coiled regions denoted alphabetically from A to H, connected by non-helical bends often involving proline. The periodicity of non-polar residues in the helical regions is 3.6 and this fact coupled with a very specific folding of the peptide chain common to all species studied, results in a subunit interior which is made up of non-polar residues, almost everywhere in van der Waals contact with their neighbours (Kendrew, 1962). Many of the glycines and alanines, being only weakly hydrophobic, lie on the surface of the molecule. The few larger non-polar side-chains which are not interior bury themselves in crevices near the surface for minimum contact with the polar environment, or lie in the boundaries between unlike subunits (Perutz, 1965). Perutz has also noted that all side-chains which are ionized at neutral pH lie at the surface of the subunits as do all but two of the polar side-chains which are hydrogen-bonded internally. The subunit surface,

then, is studded almost exclusively with charged and polar groups most of which make contact with water rather than with other polar groups. Some serve as links between the subunits.

Each of the subunits contain a heme group consisting of protoporphyrin IX with a centrally placed ferrous atom. Four of the coordination positions of the iron are occupied by the nitrogen atoms in the planar ring, the fifth by a buried histidine residue while the sixth is available for reversible binding of a ligand. Consistent with the above, the heme is contained in a non-polar pocket near the subunit surface to facilitate the uptake and discharge of oxygen and to allow the propionic side-chains to project outward and interact with the surrounding water or form salt bridges with lysines. The hemes are shown as discs in the model of Figure 1, and their structure is shown in Figure 2.

Dayhoff (1969) has tabulated the known primary structure of human hemoglobin and that of many other species, and discusses sequence as it relates to evolution. The complete sequence of human α and β subunits are presented in Figure 3.

Contacts between like subunits in the tetramer are few and limited to polar interactions near the α-amino and carboxyl termini. No non-polar groups are present on these surfaces of the subunits.

Contacts between like subunits are of two types as

Figure 2: The molecular structure of heme (Fischer and Orth, 1937; Lehmann and Huntsman, 1966).

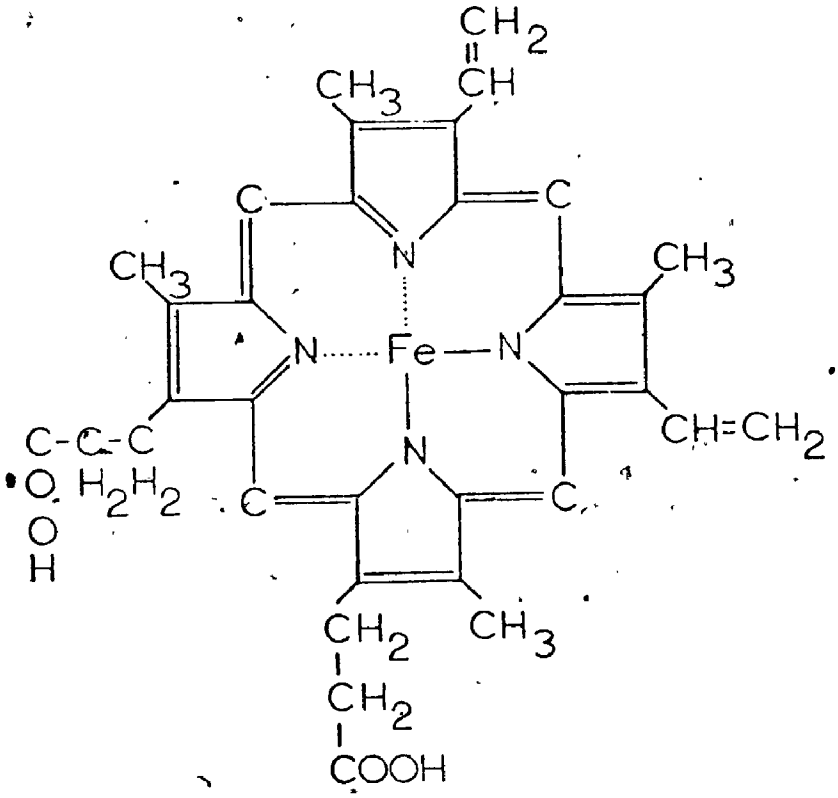
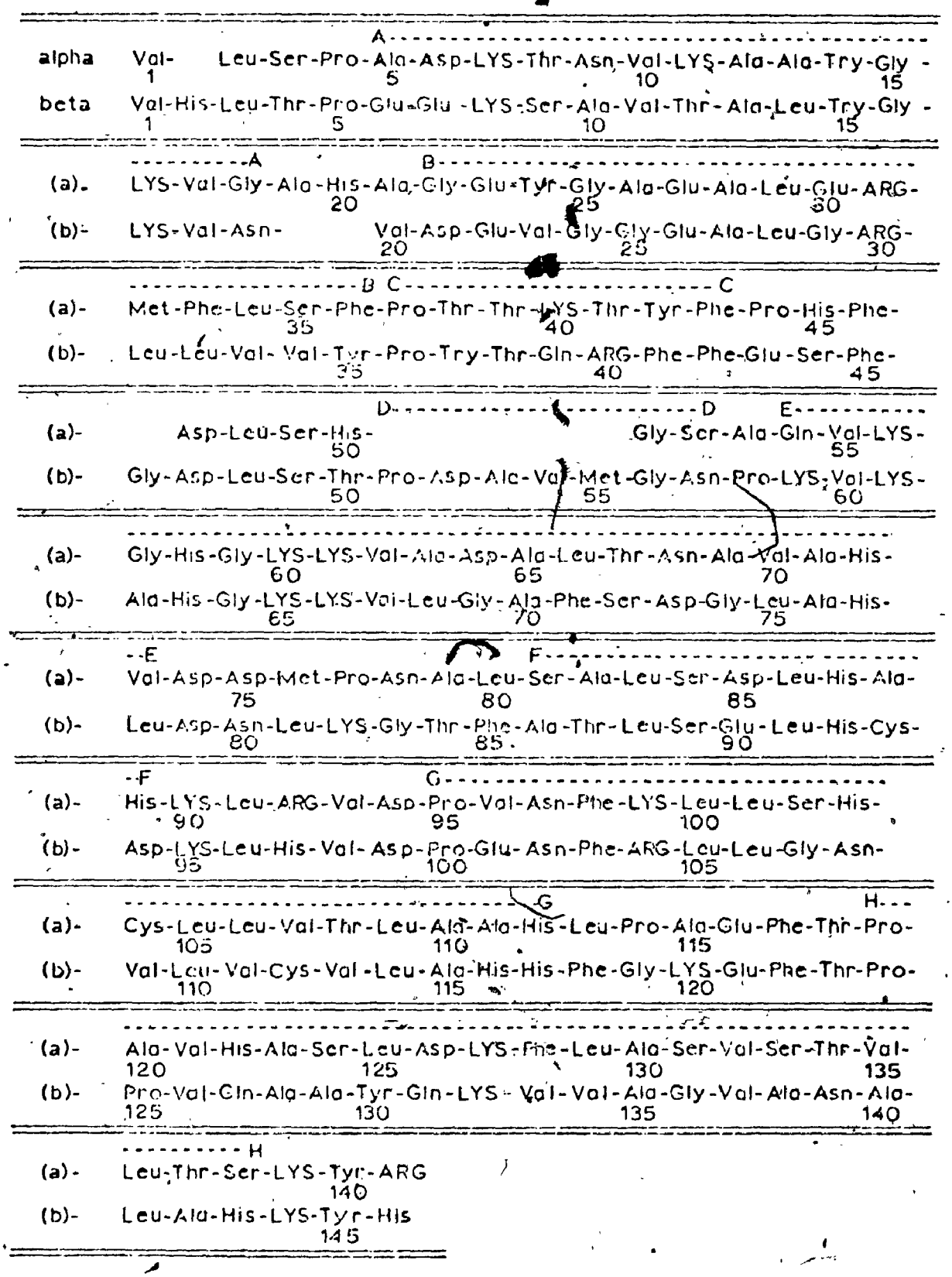
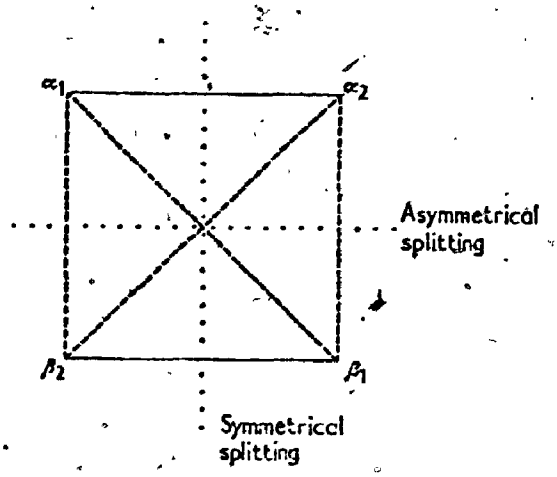

CH2
CH
CH3
C
C
N
CH3
CH3
N
Fe
N
CH=CH2
C-C-C
O H2H2
O
H
C
C
N
CH2
CH3
CH2
COOH

Figure 3: Amino acid sequence of the α and β subunit of human hemoglobin (Dayhoff, 1969).
Drawing from Oda (1969).


alpha Val- Leu-Ser-Pro-Ala-Asp-LYS-Thr-Asn-Val-LYS-Ala-Ala-Try-Gly -
beta Val-His-Leu-Thr-Pro-Glu-Glu -LYS-Ser-Ala-Val-Thr- Ala-Leu-Try-Gly -
(a). LYS-Val-Gly-Ala-His-Ala-Gly-Glu-Tyr-Gly-Ala-Glu-Ala-Leu-Glu-ARG-
(b)- LYS-Val-Asn- Val-Asp-Glu-Val-Gly-Gly-Glu-Ala-Leu-Gly-ARG-
(a)- Met-Phe-Leu-Ser-Phe-Pro-Thr-Thr-LYS-Thr-Tyr-Phe-Pro-His-Phe-
(b)- Leu-Leu-Val- Val-Tyr-Pro-Try-Thr-Gln-ARG-Phe-Phe-Glu-Ser-Phe-
(a)- Asp-Leu-Ser-His- Gly-Ser-Ala-Gln-Val-LYS-
(b)- Gly-Asp-Leu-Ser-Thr-Pro-Asp-Ala-Val-Met-Gly-Asn-Pro-LYS-Val-LYS-
(a)- Gly-His-Gly-LYS-LYS-Val-Ala-Asp-Ala-Leu-Thr-Asn-Ala-Val-Ala-His-
(b)- Ala-His-Gly-LYS-LYS-Val-Leu-Gly-Ala-Phe-Ser-Asp-Gly-Leu-Ala-His-
(a)- Val-Asp-Asp-Met-Pro-Asn-Ala-Leu-Ser-Ala-Leu-Ser-Asp-Leu-His-Ala-
(b)- Leu-Asp-Asn-Leu-LYS-Gly-Thr-Phe-Ala-Thr-Leu-Ser-Glu-Leu-His-Cys-
(a)- His-LYS-Leu-ARG-Val-Asp-Pro-Val-Asn-Phe-LYS-Leu-Leu-Ser-His-
(b)- Asp-LYS-Leu-His-Val-Asp-Pro-Glu-Asn-Phe-ARG-Leu-Leu-Gly-Asn-
(a)- Cys-Leu-Leu-Val-Thr-Leu-Ala-Ala-His-Leu-Pro-Ala-Glu-Phe-Thr-Pro-
(b)- Val-Leu-Val-Cys-Val-Leu-Ala-His-His-Phe-Gly-LYS-Glu-Phe-Thr-Pro-
(a)- Ala-Val-His-Ala-Ser-Leu-Asp-LYS-Phe-Leu-Ala-Ser-Val-Ser-Thr-Val-
(b)- Pro-Val-Gln-Ala-Ala-Tyr-Gln-LYS-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-
(a)- Leu-Thr-Ser-LYS-Tyr-ARG
(b)- Leu-Ala-His-LYS-Tyr-His


α_1
α_2
Asymmetrical splitting
β_2
β_1
Symmetrical splitting

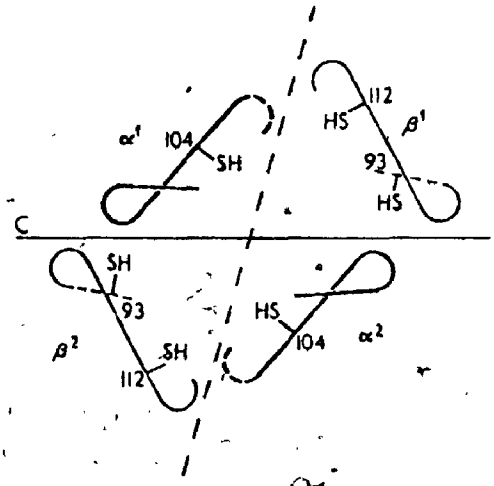
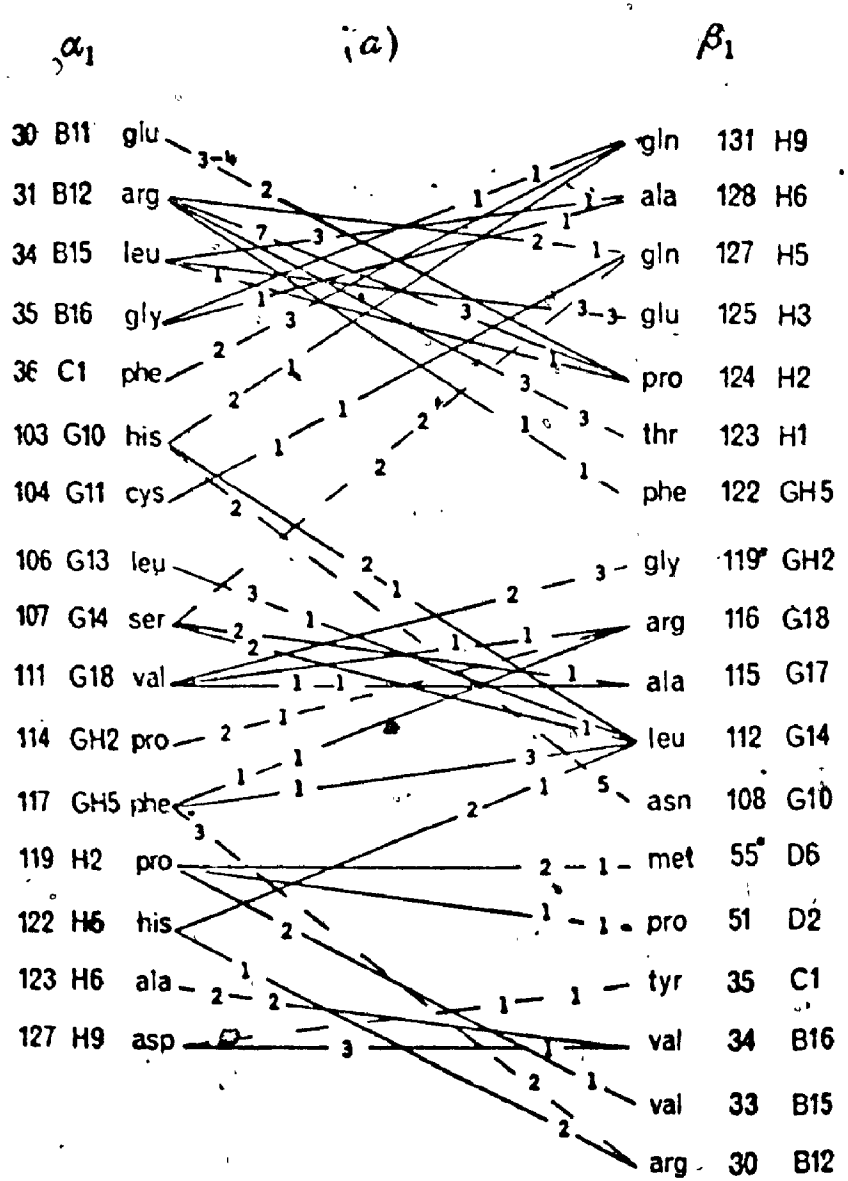
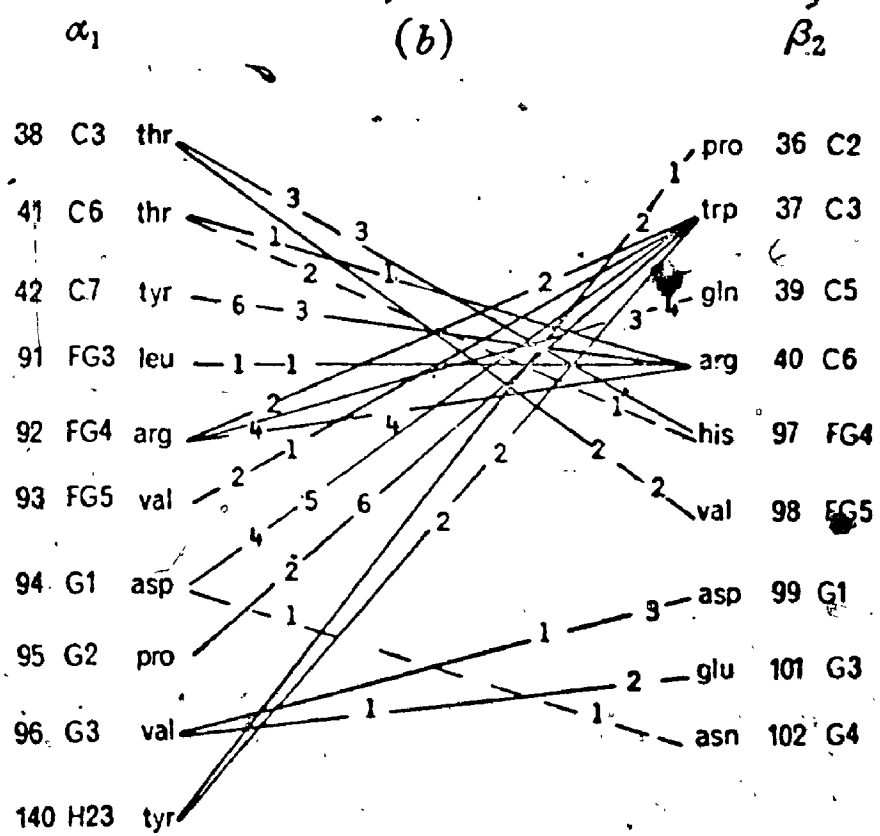

α^1
104
SH
112
HS
β^1
93
HS
C
SH
93
β^2
SH
112
HS
104
α^2

Figure 4: Amino acids which participate in the hemoglobin $\alpha_1\beta_1$ and $\alpha_1\beta_1$ contact surfaces (Perutz, 1969). Numbers on lines represent the number of atoms contributed to the contact by the nearer member of each pair joined by a line. A contact is defined as an approach to within 4 Å or less.


α1
(a)
β1
30 B11 glu
31 B12 arg
34 B15 leu
35 B16 gly
36 C1 phe
103 G10 his
104 G11 cys
106 G13 leu
107 G14 ser
111 G18 val
114 GH2 pro
117 GH5 phe
119 H2 pro
122 H6 his
123 H6 ala
127 H9 asp
gln 131 H9
ala 128 H6
gln 127 H5
glu 125 H3
pro 124 H2
thr 123 H1
phe 122 GH5
gly 119 GH2
arg 116 G18
ala 115 G17
leu 112 G14
asn 108 G10
met 55 D6
pro 51 D2
tyr 35 C1
val 34 B16
val 33 B15
arg 30 B12


α1
(b)
β2
38 C3 thr
41 C6 thr
42 C7 tyr
91 FG3 leu
92 FG4 arg
93 FG5 val
94 G1 asp
95 G2 pro
96 G3 val
140 H23 tyr
pro 36 C2
trp 37 C3
gln 39 C5
arg 40 C6
his 97 FG4
val 98 FG5
asp 99 G1
glu 101 G3
asn 102 G4

would not be expected to result in significant tertiary or quaternary structural changes.

The hemoglobin molecule in solution involves a dynamic equilibrium between tetramer and dimers and perhaps to a very limited extent, monomers. Several factors such as the nature of the ligand bound to the heme, and the concentration of hemoglobin, of neutral salt, and of hydrogen ion, affect the position of the equilibrium. In 1964, both Wyman as well as Rossi-Fanelli, Antonini and Caputo reviewed these phenomena. Since then, others have investigated the effects of a wider range of salts as well as urea and guanidine hydrochloride.

Kawahara, Kirshner and Tanford (1965) have shown that urea greatly decreases the sedimentation coefficient of hemoglobin and that a limiting value indicating complete dissociation to dimers is assymptotically approached at high urea concentrations. This process is reversible and occurs without appreciable unfolding or other conformational change. A similar effect was seen with 0.7 M GuHCl with no indication of further dissociation to monomers or of dimer instability up to 2.5 M GuHCl. Above this concentration, extensive unfolding, heme loss, and covalent aggregation involving disulfide bridge formation was observed.

Dissociation of the tetramer to half-molecules does not reach completion in NaCl due to denaturation above a

molarity of three. However a limiting $S_{20,w}^{\circ}$ value of 2.8 was reached with $CaCl_2$ and $MgCl_2$ indicating complete dissociation. This compares with values of 4.6 for hemoglobin present mainly as a tetramer at a protein concentration of 0.4 g/dl (Kawahara, Kirshner and Tanford, 1965) and 1.8 for monomer at 0.2 g/dl (Bucci et al, 1965).

Dissociation of dimer into monomers has been reported under physiological conditions of pH and electrolyte concentration by Guidotti, Konigsberg and Craig (1963) by suitably sensitive methods. Ackers and Thompson (1965) found no dissociation beyond dimer either by porous disc diffusion or by gel filtration but Schachman and Edelstein (1966) have reported the formation of monomers by oxyhemoglobin as seen in the scanning ultracentrifuge. Chiancone, Gilbert, Gilbert and Kellet (1968) have been unable to resolve this question with gel filtration plateau studies. Bucci (1971) was unable to detect dissociation to monomers in the 10^{-8} M range above pH 5.0 by complement fixation techniques, as were Kellett and Schachman (1971) by sedimentation equilibrium. Prevailing opinion is that at neutral pH and in moderate ionic strength dissociation probably occurs upon sufficient dilution but that this is beyond the capabilities of the methods available. Within the limits of concentration where measurements are possible, monomer formation is negligible. The presence of appreciable concentrations of monomers below pH 4.9 and above

pH 11.0 has been suggested (Guidotti, Konigsberg and Craig, 1963) but has been challenged at least in the upper region by Gottlieb, Robinson and Itano (1967). Monomer stability in these extreme pH ranges is, in any case, uncertain.

Asymmetrical dissociation as represented by $\alpha_2\beta_2 \rightleftarrows \alpha_2 + \beta_2$ has been discounted by the finding of Charlwood, Gratzer and Beaven (1960) that alkali-induced denaturation of fetal hemoglobin, $\alpha_2\gamma_2$, obeys first order kinetics. This would presumably not be the case if dissociation had formed the alkali labile α_2 and the alkali resistant γ_2 dimers. In addition, Vinograd and Hutchinson (1960) have detected a significant time lag prior to the appearance of hybrid molecules even when the hemoglobins are electrolyte-dissociated at the time of mixing. Perutz has neatly explained the absence of asymmetrical dissociation by noting that it would require cleavage of both pairs of strong non-polar contacts and neither of the weak polar contacts. See Figure 5 (Perutz, 1965). A schematic diagram of Rosemeyer and Huehns (1967) further illustrates the dissociation modes (Figure 6). All of the evidence indicates symmetrical dissociation, $\alpha_2\beta_2 \rightleftarrows 2\alpha\beta$, which involves cleavage of only one of the non-polar contacts and the two polar contacts. It is therefore more meaningful to denote hemoglobin as $(\alpha\beta)_2$ than as $\alpha_2\beta_2$.

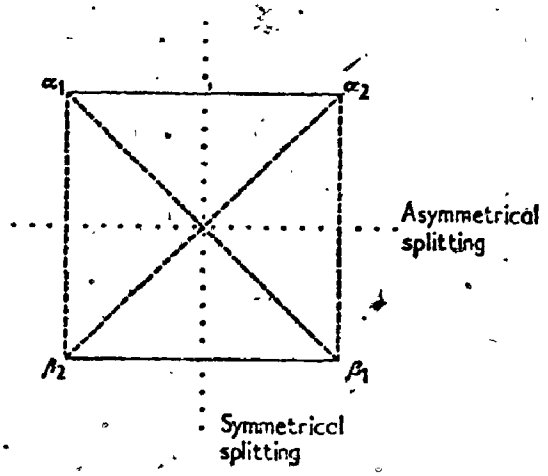
The type of $\alpha\beta$ dimer formed has been the subject

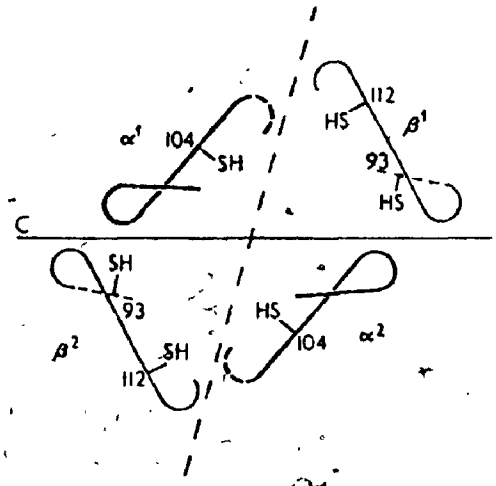
Figure 5: Modes of hemoglobin cleavage (Perutz, 1965).

(——) Polar contacts.

(---) Non-polar contacts.

Figure 6: A second illustration of hemoglobin cleavage (Rosemeyer and Huehns, 1967).


α_1
α_2
Asymmetrical splitting
β_2
β_1
Symmetrical splitting


112
HS
β^1
α^1
104
SH
93
HS
C
SH
93
HS
β^2
SH
104
α^2
112

of recent debate. Kawahara et al (1965) have concluded that one of the α-β "bonds" is weak relative to the other, that the forces involved in this contact site are not primarily hydrophobic, and that all dissociating agents act upon this bond. Perutz has stated that symmetrical dissociation occurs exclusively along the less extensive non-polar contact giving rise to $\alpha_1\beta_1$ dimers only (Perutz, 1970a). However Edelstein et al (1970) have suggested that the difference in total bonding strength involved in the two contact areas, based on 110 atomic interactions in the case of the $\alpha_1\beta_1$ interface compared with 80 for $\alpha_1\beta_2$, is not sufficient to rule out formation of the $\alpha_1\beta_2$ dimer. They sought to support this view with evidence that deoxyhemoglobin dissociates to form a dimer which reacts with carbon monoxide at a rate different from that of the dimer formed by dissociation of liganded Hb. They inferred that this difference in reactivity could be due to the formation of $\alpha_1\beta_2$ rather than $\alpha_1\beta_1$ dimers. Perutz has cast serious doubts on this evidence by quoting a private communication from Kellet, Midgarden, and Schachmann who found that "the deoxy form remains tetrameric in all conditions formerly believed to produce dimers" (Perutz, 1970a).

Rosemeyer and Huehns (1967) strongly support the proposal of $\alpha_1\beta_2$ interface cleavage on the basis of data involving non-reversible dissociation due to covalent

modification of sulfhydryl groups exposed to the reagent PCMB upon electrolyte-induced dissociation. The dimeric state is maintained following rapid reaction of β93 -SH, situated near the $\alpha_1\beta_2$ contact. Further dissociation to monomers is not observed until the virtually unreactive β-112 and α-104 sulfhydryls become modified. These two are known to be in the $\alpha_1\beta_1$ contact area (Muirhead and Perutz, 1963; Perutz, 1965).

Therefore it can be concluded that symmetrical dissociation of the hemoglobin tetramer occurs to produce two $\alpha_1\beta_1$ dimers, with little or no formation of monomers which is unaccompanied by denaturation.

Hemoglobin Function

The physiological role of hemoglobin is to transport molecular oxygen from the lungs to the peripheral tissues and to assist in the removal and expiration of carbon dioxide generated upon the metabolic use of oxygen. Hemoglobin is well suited for this role due to an allosteric nature of ligand binding. A plot of fractional saturation with oxygen versus partial pressure of oxygen results in a sigmoid curve which can be approximated by the Hill equation (Hill, 1910):

$$Y = \frac{K \cdot p^n}{1 + Kp^n}$$

where Y is the fractional saturation, p is the O_2 partial pressure, and K and n are empirical constants. The system can be written as $Hb + nO_2 \rightleftarrows Hb\ (O_2)_n$. Oxygen affinity of various types of Hb is often estimated by measurement of $P_{\frac{1}{2}}$, the partial pressure required to half-saturate a sample of hemoglobin. The value of n is a measure of the allosteric interaction between subunits and has a value of 2.7 for human Hb.

Although the partial pressure of oxygen in the tissues is not much lower than that in the lungs, the allosteric behavior resulting from subunit-subunit interaction, commonly referred to as heme-heme interaction, ensures an increased affinity as successive molecules of oxygen bind and causes the retentive affinity to fall as successive molecules dissociate. The affinity of isolated α and β subunits for oxygen equals that of fully saturated tetrameric hemoglobin. The sigmoid nature of binding is therefore due to constraints present in the tetramer. The conformational changes involved are extensive and are triggered by the movement of the heme iron atom in the plane of the porphyrin ring.

The effectiveness of hemoglobin is enhanced by both the Bohr effect and the stereo-cooperativity of 2,3-diphosphoglycerate. Hemoglobin also functions in the reverse transport of carbon dioxide. Human oxyHb binds about 0.15 mMoles CO_2 per mole of heme while deoxyHb binds

0.04 mMoles. Carbon dioxide is removed from the tissues indirectly by the bicarbonate buffering system.

The Bohr effect, which refers to the uptake of protons by hemoglobin upon the liberation of ligand, amplifies the effectiveness of this bicarbonate system. The fact that the functional unit is the tetramer rather than the dimer coupled with the fact that the Bohr effect is not present in Hb bound to haptoglobin is of interest and will be expanded upon in the discussion following.

The organic phosphate 2,3-diphosphoglycerate, present in red cells, binds specifically in a 1:1 molar ratio to deoxyhemoglobin in the central cavity thereby increasing the free energy of heme-heme interaction and lowering the affinity for oxygen in the tissues. This binding does not affect the Bohr effect. Upon oxygenation, the 2,3-diphosphoglycerate is expelled, the binding site is lost. It also does not, therefore, affect deoxygenation.

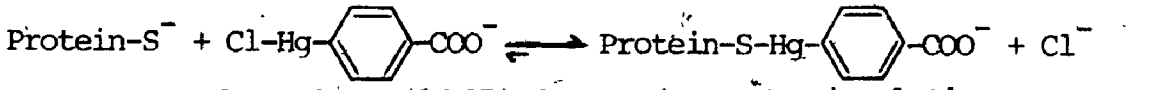
It is not possible to do justice to these topics in the space here available. The reader is therefore referred to the detailed discussions and explanations of these effects on a molecular level as presented by Perutz (1965; 1970 a, b).

HEMOGLOBIN SUBUNITS

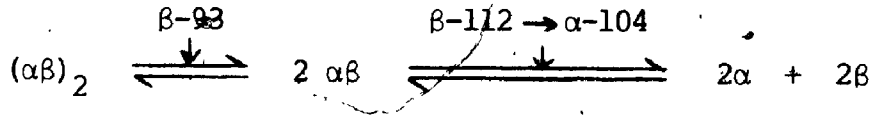
The evolution of methods designed to isolate the α and β subunits of human hemoglobin was begun by Wilson

and Smith (1959) and Hill and Craig (1959). Both involved separation with denaturation. It progressed through a method yielding the cyanmet derivative which ruled out any functional studies (Huehns, Dance and Shooter, 1962) and those based on the greater acid stability of α compared to β allowing isolation of the former at the expense of precipitation of the latter (Huehns et al, 1961; Ranney, Briehl and Jacobs, 1965) towards preparations which would be at once native, stable and pure.

A major advancement came with the splitting by Bucci and Fronticelli (1965) of hemoglobin whose dissociation was prompted by moderate ionic strength. Formation of separate subunits followed sulfhydryl modification with p-chloromercuribenzoate (PCMB) according to the reaction



Rosemeyer and Huehns (1967) have characterized the process as



Reaction at β-93 occurs rapidly and enhances formation of dimers. The S value of dimer is assymptotically approached as the molar ratio of reagent to hemoglobin is increased from zero to four. Introduction of the bulky negative PMB group at β-112 makes modification of α-104 possible. When all three -SH have become -S-PMB association to any state above monomers is slight. Bucci and Fronticelli then separated the PMB-subunits by pH

gradient elution from a CM-cellulose column. The profile was sometimes complicated by small amounts of undissociated hemoglobin and additional peaks regarded as artefacts. Regeneration of the sulfhydryls was by prolonged dialysis against thioglycolate or cysteine. DeRenzo et al (1967) sought to shorten the regeneration time of thirty hours (which included that of dialysis removal of regenerating agent) by emulsification with the water insoluble 1-dodecanethiol. The subunits produced were essentially homogeneous upon electrophoresis and sedimentation analysis but were prone to precipitation from solution probably resulting from thiol binding.

A more satisfactory method was proposed by Geraci, Parkhurst and Gibson (1969). Chain separation was accomplished by DEAE-cellulose chromatography. The α^{PMB} subunits, which did not bind, were washed out. Then column treatment with β mercaptoethanol (βME) both regenerated the β sulfhydryls and changed the subunit net charge thereby causing elution. In a like manner, the PMB was removed from α subunits bound to CM-cellulose.

Sulfhydryl titration by the method of Boyer (1954) indicated complete -SH regeneration of the α subunit but not of the β. Thus further methods were sought to render the latter to their native state. The need to prepare β for isolated subunit studies was not as pressing due to its limited availability as hemoglobin H (β_4).

Tyuma, Benesch and Benesch (1966) had developed a quite effective method involving passage through a sulfhydryl Sephadex column. Kilmartin and Fogg (1973) have more recently suggested retention of the simpler treatment of Geraci et al (1969) but with protection of the β subunit from βME-induced denaturation by prior conversion to the carbon monoxide derivative so that higher concentrations of βME can be used. This ensures complete removal of the PMB. Replacement of the CO with O_2 can be carried out as desired (Kilmartin and Rossi-Bernardi, 1971).

The structures of the α and β subunits are similar but not identical. Perutz (1965) has detailed by X-ray crystallography the great similarity especially in the region of the heme. Although there is no significant difference in the visible spectrum of isolated and tetrameric subunits (Antonini, Bucci, et al, 1965), their conformations are not the same since the molar intensity of circular dichroism for hemoglobin is not the sum of that possessed by the isolated subunits (Nagai et al, 1969) This implies conformational changes as the tetramer structure is assumed (Nagai et al, 1969). Several studies designed to detect structural changes upon the binding of ligand to isolated subunits have been negative (Perutz and Mazzarella, 1963; Chiancone, Alfsen et al, 1968) but McDonald and Noble (1974) have indicated that a transition does occur with native β but not α as detected by changes in reactivity with PCMB.

haptoglobin: 1-1, 1-2, 2-2. All other vertebrates studied to date have but one Hp type, similar to human 1-1 (Jayle and Moretti, 1962) and consist of two pairs of polypeptide chains denoted as light, L, and heavy, H, or α and β. Their individual molecular weights are 8,900 ± 400 and 40,000-43,000 (Connell, Smithies and Dixon, 1966) resulting in a total molecular weight of 100,000 ± 2,000 for the intact Hp 1-1 molecule. Cheftel and Moretti (1966) have confirmed this by reporting a molecular weight of 98,700 determined by equilibrium centrifugation.

Following reduction and alkylation, Shim and Bearn (1964) separated the chains by Sephadex chromatography and noted that the carbohydrate content was confined to the heavy chain only and made up 20% of its molecular weight. They proposed a structural model involving three disulfide bridges and analogous to that of the immunoglobins.

There is considerably more information available on the light than heavy chain. The sequence has been determined by Black and Dixon (1968) (Figure 7) indicating three cysteine residues two of which were later reported to form an intra-chain loop and the third to participate in a symmetrical disulfide bridge between the two L chains (Black and Dixon, 1970). These data did not make provision for any sulfhydryls which could be involved in

seen by the lack of recombination ability and partial unfolding of the remaining subunits to some intermediate state could not be ruled out.

Bucci et al (1965) also reported association of α^{SH} on the basis of sedimentation velocity dependence on concentration. The subunits, prepared by the method of Bucci and Fronticelli (1965) were homogeneous in the ultracentrifuge and on Sephadex but were seen to consist of three components when examined by the more sensitive method of starch gel electrophoresis.

Rosemeyer and Huehns (1967) found no formation of an intermediate by normal and abnormal α^{PMB} subunits (hemoglobin A and J) and concluded that they are "entirely monomeric [as seen by this technique]" but with reference to Bucci et al (1965) spoke of the subunits as being "largely monomeric".

The functional properties of the subunits change drastically upon isolation. In direct contrast to hemoglobin, they have high oxygen affinity, and lack heme-heme interaction and the Bohr effect (Ranney et al, 1965; Ranney et al 1961). Evidently interaction between unlike subunits is necessary for functionality.

HAPTOGLOBIN

Detection and Assay

Haptoglobin is an α_2-globulin serum glycoprotein whose most striking characteristic is a great affinity

and specificity for binding hemoglobin. This binding to form a hemoglobin haptoglobin complex is accompanied by a stabilization of the participating hemoglobin molecule in acid conditions (Sasazuki et al, 1973) which is observed as an enhancement of peroxidase activity. This led to its discovery by Polonovski and Jayle (1938). Jayle (1951) later used this enhancement as the basis of an assay for haptoglobin. More recent assay methods take advantage of the strength of binding and the 1:1 stoichiometry involved. The haptoglobin present can be titrated with hemoglobin to an end point indicated by no further fluorescence quenching of aromatic residues of haptoglobin. This can be done more conveniently by the addition of an amount of Hb greater than that required to saturate the Hp present followed by determination of the excess.

The formation and presence of the hemoglobin haptoglobin complex has been detected by measurement of peroxidase activity (Connell and Smithies, 1959), as well as by electrophoresis (Bernier, 1967), gel filtration (Killander, 1964; Ratcliff and Hardwicke, 1964) and by protection of complexed hemoglobin against the radical conformational changes normally induced by pH adjustment to 3.7 (Roy, Shaw and Connell, 1969).

Haptoglobin Structure

By starch gel electrophoresis, Smithies and Walker (1956) distinguished the three genetic variants of human

haptoglobin: 1-1, 1-2, 2-2. All other vertebrates studied to date have but one Hp type, similar to human 1-1 (Jayle and Moretti, 1962) and consist of two pairs of polypeptide chains denoted as light, L, and heavy, H, or α and β. Their individual molecular weights are 8,900 $\pm$ 400 and 40,000-43,000 (Connell, Smithies and Dixon, 1966) resulting in a total molecular weight of 100,000 $\pm$ 2,000 for the intact Hp 1-1 molecule. Cheftel and Moretti (1966) have confirmed this by reporting a molecular weight of 98,700 determined by equilibrium centrifugation.

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Figure 7: Amino acid sequence of light chain of human haptoglobin 1-1 (Black and Dixon, 1968).

10

H_2N-Val-Asn-Asp-Ser-Gly-Asn-Asp-Val-Thr-Asp-Ile-Ala-Asp-

20

Asp-Gly-Gln-Pro-Pro-Pro-Lys-Cys-Ile-Ala-His-Gly-Tyr-Val-

30 40

Glu-His-Ser-Val-Arg-Tyr-Gln-Cys-Lys-Asn-Tyr-Tyr-Lys-Leu-

50

Arg-Thr-Gln-Gly-Asp-Gly-Val-Thr-Thr-Leu-Asn-Asn-Lys-Lys-

60

Gln-Trp-Ile-Asn-Lys-Ala-Val-Gly-Asp-Lys-Leu-Pro-Glu-Cys-

70 80

Glu-Ala-Val-Gly-Lys-Pro-Lys-Asn-Pro-Ala-Asn-Pro-Val-Gln-

COOH

a link to the H chains. However, communications from the Dixon laboratory (Malchy and Dixon, 1973 a, b) recently indicated the existence of a fourth cysteine residue at position 73 which could bridge to the H chain.

Both porcine (Fraser and Smith, 1971) and human (Bernini and Borri-Voltattorni, 1970) reduced Hp 1-1 have 18 sulfhydryl groups, none of which occur as -SH in the native molecule (Lisowska and Dobryszcka, 1967). Tattrie and Connell (1967) found less than one sulfhydryl reactive to ^{14}C-iodoacetate or p-chloromercuribenzoate in 7.2 M guanidine hydrochloride. Bernini and Borri-Voltattorni (1970) found none reactive to 5,5'-dithiobis-(2-nitrobenzoic acid) in either 6 M GuHCl or 33 mM SDS. The ten free -SH groups seen by Waks and Alfsen (1966) in the presence of 8 M urea are probably due to inadvertant reduction to which Hp is known to be very susceptible (Lockhart and Smith, 1971). Thus all 18 cysteine residues, four of which are present in each of the L chains, are involved in disulfide bonds.

The concentration and pH-dependent dissociation of Hp reported by Waks and Alfsen (1968) has been refuted by the gel filtration and sedimentation velocity work of Lockhart, Chung and Smith (1972), consistent with the prevailing opinion that all chains are covalently linked.

The carbohydrate moiety of Hp was initially reported to contribute 11% of the molecular weight (Jayle, Boussier

and Tonnelot, 1956) while a more recent estimate indicates 18.6% (Schultze et al, 1963 ; Cheftel et al, 1965), all of which is attached to the H chains (Shim and Bearn, 1964; Cheftel and Moretti, 1966; Lisowska and Dobryszycka, 1967). Position of attachment and sequence have not yet been established but there are thought to be eight chains consisting of galactose, mannose and glucosamine residues with two of the chains being terminal in sialic acid (Cheftel et al, 1965).

Contrary to the structural model of Shim and Bearn, Malchy and Dixon (1973 a) have found that the L chains rather than the H chains are linked by disulfide bridges and have proposed a revised model of Hp 1-1. Figure 8 incorporates all present knowledge. Lockhart, Chung and Smith (1972) have confirmed this arrangement by identifying Hp fragments produced upon progressive disulfide cleavage with β-mercaptoethanol.

The three genetically determined types of human haptoglobin result from three different L chains, denoted as α^{1F}, α^{1S}, and α^{2}. The first two are thought to be identical except at position 54 (LYS and GLU respectively), while α^{2} consists of both of them but with one segment deleted, as shown in Figure 9 (Smithies, Connell, and Dixon, 1962; Black and Dixon, 1968). The proposed chain compositions of the variants are: Hp 1-1 as $(\alpha^{1}\beta)_2$, Hp 2-1 and $(\alpha^{1}\beta)_2(\alpha^{2}\beta)n$ where n = 0, 2, 4,...,

Figure 8: A model of Hp 1-1 based on present knowledge.

Figure from Oda (1972).

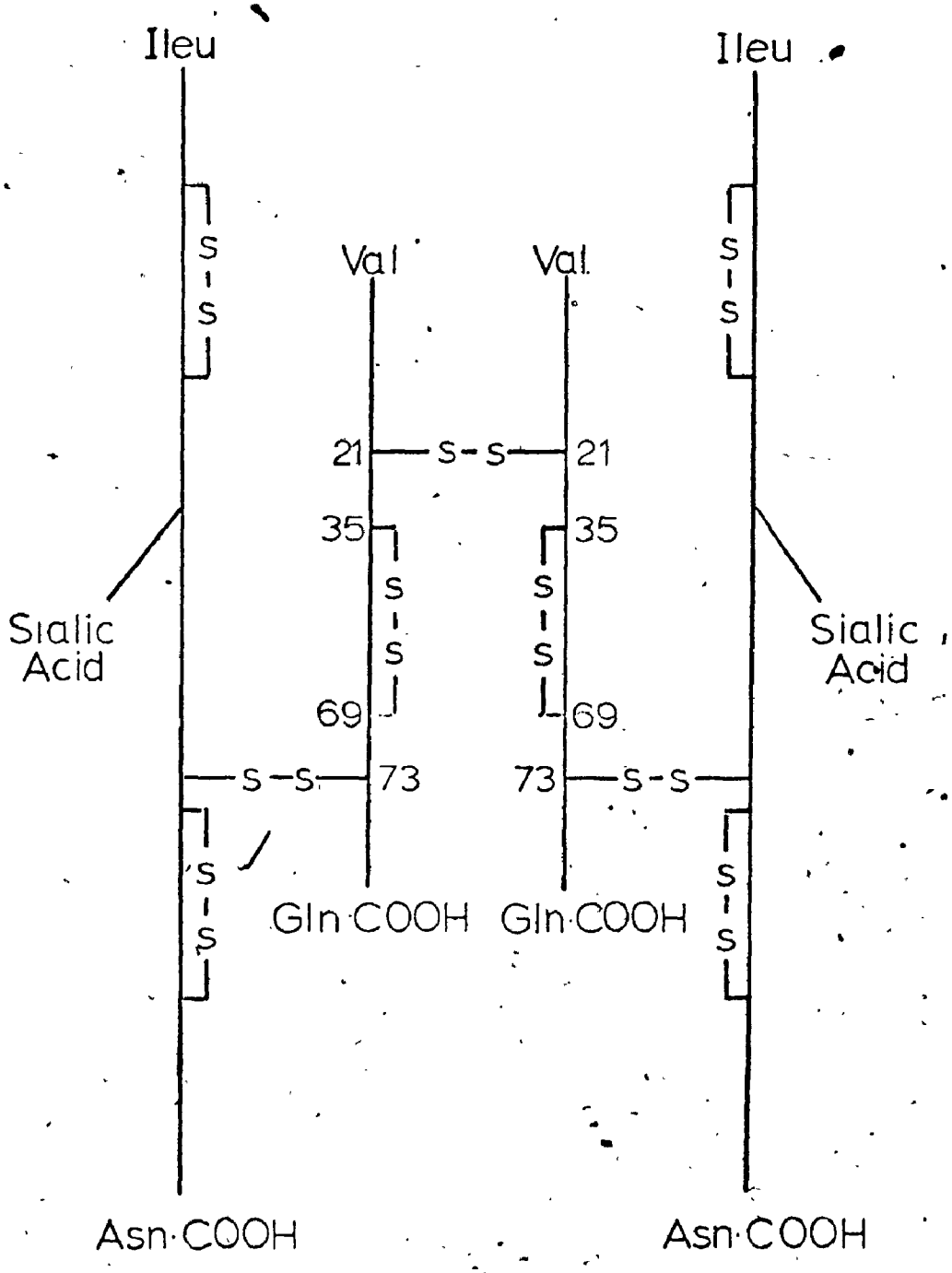

Ileu
Ileu
S-S
S-S
Val
Val
21
S-S
21
35
35
S-S
S-S
Sialic Acid
Sialic Acid
69
69
S-S
73
73
S-S
S-S
S-S
Gln·COOH
Gln·COOH
Asn·COOH
Asn·COOH

Figure 9. The amino acid sequence of human haptoglobin α^S and α^2 chain.

Haptoglobin α^S

NH_2-Val-Asn-Asp-Ser-Gly-Asn-Asp-Val-Thr-Asp-Ile-Ala-Asp-Asp-Gly-Gln-Pro-Pro-Pro-Lys-
-Cys-Ile-Ala-His-Gly-Tyr-Val-Glu-His-Ser-Val-Arg-Tyr-Gln-Cys-Lys-Asn-Tyr-Tyr-Lys-
-Leu-Arg-Thr-Gln-Gly-Asp-Gly-Val-Tyr-Thr-Leu-Asn-Asn-Glu-Lys-Gln-Trp-Ile-Asn-Lys-
-Ala-Val-Gly-Asp-Lys-Leu-Pro-Glu-Cys-Glu-Ala-Val-Gly-Lys-Pro-Lys-Asn-Pro-Ala-Asn-
-Pro-Val-Gln-COOH

Haptoglobin α^2

NH_2-Val-Asn-Asp-Ser-Gly-Asn-Asp-Val-Thr-Asp-Ile-Ala-Asp-Asp-Gly-Gln-Pro-Pro-Pro-Lys-
-Cys-Ile-Ala-His-Gly-Tyr-Val-Glu-His-Ser-Val-Arg-Tyr-Gln-Cys-Lys-Asn-Tyr-Tyr-Lys-
-Leu-Arg-Thr-Gln-Gly-Asp-Gly-Val-Tyr-Thr-Leu-Asn-Asn-Lys/Glu-Lys-Gln-Trp-Ile-Asn-Lys-
-Ala-Val-Gly-Asp-Lys-Leu-Pro-Glu-Cys-Glu-Ala-Asp-Asp-Gly-Gln-Pro-Pro-Pro-Lys-Cys-
-Ile-Ala-His-Gly-Tyr-Val-Glu-His-Ser-Val-Arg-Tyr-Gln-Cys-Lys-Asn-Tyr-Tyr-Lys-Leu-
-Arg-Thr-Gln-Gly-Asp-Gly-Val-Tyr-Thr-Leu-Asn-Asn-Lys/Glu-Lys-Gln-Trp-Ile-Asn-Lys-Ala-
-Val-Gly-Asp-Lys-Leu-Pro-Glu-Cys-Glu-Ala-Val-Gly-Lys-Pro-Lys-Asn-Pro-Ala-Asn-Pro-
-Val-Gln-COOH

and Hp 2-2 as $(\alpha^2\beta)_n$ where n = 4,6,8,... (Shim and Bearn, 1964). The method of polymerization has been partially elucidated (Malchy and Dixon, 1973 a).

In the present work, the inconvenience of obtaining sufficient plasma of 1-1 homozygous individuals or separating Hp 1-1 from polymeric species in the plasma of heterozygotes has been avoided by the use of porcine haptoglobin which is known to closely resemble human Hp 1-1 in both molecular weight, amino acid composition, and ability to bind human Hb (Fraser and Smith, 1971; Black, Chan, Hew and Dixon, 1970).

Haptoglobin Binding Site for Hemoglobin

The site of hemoglobin binding to Hp involves primarily the H chain. The hemoglobin binding ability of the individual chains isolated from reduced alkylated Hp 2-2 on Sephadex G-100 in dilute propionic acid by Gordon and Bearn (1966) was considerable for the H chain but minimal for the L chain. Using a second method of chain isolation involving G-200 Sephadex in 5 M GuHCl Gordon, Cleave and Bearn (1968) reported further supportive evidence indicating that the H chain makes the major contribution to the Hp binding site for hemoglobin.

Ofusu, Cambell and Connell (1971) have obtained two plasmin digestion fragments of Hp 1-1 with molecular weights of 78,000 and 17,000. Since the amino-termini of the larger were the same as those of intact Hp, that is,

valine and isoleucine, this fragment probably involved the two L chains disulfide-bridged to the H chains but with the latter shortened by a length resulting in the smaller fragment. The cleavage position was apparently the same in the H chains as only a single amino-terminal amino acid, serine, was found in the 17,000 MW peptides. Weak binding of hemoglobin by the larger fragment was observed (Ofusu and Connell, 1971) while the smaller showed no ability to bind Hb. The fragments associated in neutral and alkaline buffer and formed a stable product upon addition of Hb which was indistinguishable from normal HpHb complex. Whether a portion of the carboxy-terminal fragment of Hp H chain makes up part of the binding site of hemoglobin or induces a more favourable conformation for binding in the larger fragment is unknown.

Haptoglobin Physiological Function

Although the total physiological role of haptoglobin is not fully understood, its involvement in the disposal of extra-corpuscular hemoglobin is certain. The molecular weight increase of hemoglobin by 100,000 daltons upon the binding of haptglobin seems sufficient to prevent its glomerular filtration (Laurell and Nyman, 1957; Allison and Rees, 1957) and thus avoids renal hemoglobin excretion and conveys protection from siderosis, produced by chronic passage of Hb through the kidneys.

The metabolic fate of the HpHb complex is catabolism

within the Kupffer cells of the liver sinusoids as well as the splenic cells (Engler, Bescol-Liversac, and Moretti, 1966; Peters and Alper, 1966). The oxidative cleavage of the four iron-containing porphyrin rings present in each Hb molecule to formylbiliverdin is catalyzed by the enzyme α-methenyl oxygenase. However this enzyme is inactive with free hemoglobin as substrate but its action is potentiated by the binding of the Hb to Hp probably due to increased ring exposure upon complex formation (Nakajima and Yamaguchi, 1962). Thus the role of haptoglobin in deciding the fate of circulating hemoglobin is to prevent its renal excretion and enable the body's catabolic mechanisms to recycle heme groups and retain iron.

An interesting side issue to this discussion is the greater activity of α-methenyl oxygenase on hemoglobin bound by Hp 2-2 than on that bound by either Hp 1-1 or Hp 2-1 reported by Nakajima et al in 1963. They have suggested that the Hp 2-2 gene may confer a selective advantage by more efficient breakdown of hemoglobin (as HpHb complex) into globin, bile pigment precursors and reusable iron. This is supported by the finding that Indian and South East Asian populations, known to have a high incidence of hemolytic disease, also have a high frequency of the Hp 2 allele. Thus the appearance of this Hp 2 allele in man may be part of a continuing evolutionary process designed to cope with the problem of hemolysis.

Additional roles of haptoglobin may include transport

of vitamin B_{12}, as suggested by Jayle and Moretti (1962) upon interpretation of the report of Latner and Zaki (1957) that isotopically labelled cobalamin was bound by α_2-plasma proteins. The clinical significance of the potent inhibition of cathepsin B by haptoglobin as seen by Snellman and Sylven (1967) has yet to be properly investigated.

Reviews of the haptoglobin literature have been made by Jayle and Moretti (1962), by Laurell and Gronwall (1962) and by Sutton (1970).

HAPTOGLOBIN HEMOGLOBIN COMPLEX

The binding of hemoglobin by haptoglobin is of such strength as to involve association constants of the order of 10^9 (Clark, 1966) and approach irreversibility. No covalent links are present but the complex is stable to conditions such as 8 M urea pH 8.0 (Chan, 1968). Extreme treatments such as exposure to dioxane or SDS (Jaenicke and Pavlicek, 1970), or to 5 M GuHCl after aminoethylation (Fraser, 1969) cause complete dissociation but are accompanied by denaturation. Less severe conditions such as 0.04 M acetic acid pH 4.7 can promote dissociation sufficiently to be detected by exchange of radioactively labelled Hb upon extended incubation (Oda, 1972). Much work has been devoted to elucidate the components and forces involved in this binding as well as to the mechanism of its formation.

That it is the globin rather than the heme portion of the participating hemoglobin molecule which is involved in complex formation is evident. The nature of the ligand or even the presence of the heme is immaterial since carboxy, cyanmet, and oxy hemoglobin complex equally well (Nagel and Gibson, 1967) as does globin (Laurell and Gronwall, 1962). In addition Hb will form complex across the wide 4.6 to 11.0 pH range, that is, where changes in the heme environment are known to occur (Nagel and Gibson, 1967).

A considerable amount of work has been designed to implicate or rule out the involvement of certain individual or groups of protein residues, so that the area of contact could be elucidated.

The involvement of amino groups of Hb was suggested by Van Royen (1950) upon observation that hemoglobin treated with formaldehyde was unable to form complex. Fraenkel-Conrat (1957), however, has shown that formaldehyde reacts with $-NH_2$ or -SH groups to produce methylol which can condense with amine, guanidyl, phenolic or heterocyclic groups to produce cross-linking methylene bridges. The loss of binding ability was therefore due to covalent constraints rather than simple amino group modification.

X-ray analysis has revealed that the lysyl residues of hemoglobin are scattered across the entire external surface of the tetramer (Perutz, 1969). Lockhart and Smith (1971) quantitatively modified the Hb lysyl residues with

ethyl acetimidate and found no change in ability to bind to haptoglobin. They therefore concluded that the surface of hemoglobin which complexes with haptoglobin lies in the interior of the tetramer. The impaired ability to form complex of haptoglobin partially trinitrophenylated with 2,4,5-trinitrobenzene-1-sulphonic acid led Shinoda (1965) to conclude that Hp amino groups were involved. This was not supported by the findings of Chan (1968) in which the extent of reaction of lysyl residues with 1-guanyl-3,5-dimethyl pyrazole in complex was within 3% of that found with hemoglobin and haptoglobin reacted separately. The area of contact between the two proteins in complex must therefore be either minimal (which is doubtful in view of the binding strength) or lysine deficient.

Kalous and Pavlicek (1965) have titrated hemoglobin, haptoglobin, and their complex. Their data showed no loss of carboxyl groups upon complex formation, thereby eliminating the possibility of their involvement, but detected the burial of twenty-six histidine residues. Loss of the enhanced peroxidase activity of hemoglobin bound to haptoglobin paralleled the selective destruction of photo-oxidation of histidine in Hb and tyrosine in Hp. However peroxidase activity is not synonymous with binding ability as shown by Dobryszcka et al (1969). When tyrosine and tryptophan residues of canine Hp were modified with

N-acetylimidazole and 2-hydroxy-5-nitrobenzyl bromide respectively, complex could be formed with hemoglobin as demonstrated by starch gel electrophoresis, but was not accompanied by hemoglobin peroxidase enhancement. Thus from these two reports it may be said that a histidine-rich area of Hb binds to Hp. The role of tyrosine residues of Hp is not clear since nitration of human Hp abolished its ability to bind as well as enhance the peroxidase activity of Hb (Chiao and Bezkorovainy, 1972). Species differences may be the cause of these diverging findings as all of the tyrosyl residues of human Hp were accessible to nitration while those in canine Hp were not.

Modification of the Hb β93 -SH with several specific reagents does not prevent complex formation (Bunn, 1967). However formation of complex involving unmodified Hb renders this group less reactive (Malchy and Dixon, 1969). This would suggest that the β93 residue is near the edge of the HbHp contact interface and that structural flexibility and the potential by many other interactions make complex formation favourable in the case of modified Hb, but once the complex is formed, optimum association is made which presumably shields the -SH.

The immunological experiments of Sasazuki et al (1974) indicated no modification or masking of the antigenic determinants of hemoglobin upon binding to haptoglobin. Since antibody is thought to interact with Hb as a

tetramer, the $\alpha_2\beta_2$ exterior surfaces are not likely to be involved in complex formation with Hp.

Jaenicke and Pavlicek (1970) have studied the effect of various dissociating media on the HpHb complex. High concentrations of urea, GuHCl, NaCl, dioxane and formaldehyde were required to induce complex dissociation but low molar ratios of complex to SDS gave the same result. This was interpreted as evidence that ion-pairs and hydrogen bonds stabilized the complex and that hydrophobic interactions were of little importance. Comparison of the heat of reaction upon complex formation with that of a similar process in β-lactoglobulin led Adams and Weiss (1969) to conclude that hydrogen bonding, reinforced by hydrophobic interactions, were involved. Based on a comprehensive study of the exchange of free and complexed hemoglobin in different environments, Oda (1972) has found that ion-pairs, H-bonds and hydrophobic interactions contribute.

The mechanism of formation and the character of the complex have also been the subject of great interest. Static and fast flow measurements of fluorescene quenching of Hp aromatic residues by the heme moiety of hemoglobin upon complex formation has been a useful technique. Nagel and Gibson (1967) found that Hp bound more rapidly to low concentrations of Hb than to high concentrations suggesting that dissociation of the tetramer was a prerequisite to binding. Varying the Hp

concentration in an excess of Hb had no effect on reaction rate. The question as to whether the reaction proceeded through consecutive binding of α and β monomers or by attachment of $\alpha_1\beta_1$ dimers could not be resolved. However the reaction kinetics of dilute Hb were similar to those seen in 2 M NaCl where Hb exists mainly as the $\alpha\beta$ dimer (Antonini et al, 1962).

These authors later investigated the rate of complex formation upon mixing deoxyHb with a carbon monoxide-saturated solution of Hp 1-1 (Nagel and Gibson, 1971). DeoxyHb is known to exist almost exclusively as a tetramer (Edelstein et al, 1970). Since conversion of deoxy to carboxyHb is much more rapid than is dissociation of tetramer to dimer (half times of about 10 msec and 1 sec respectively) a delay in quenching relative to that produced by mixing CO-Hb with Hp would indicate the necessity of dimerization prior to complexation. This time lag was in fact observed. Comparison of experimental and theoretically-generated binding curves provided additional proof of dimer rather than tetramer involvement but could not differentiate between the dimer and monomer possibilities.

Nagel and Gibson (1967) have also presented evidence that complex formation proceeds via the dimer or monomer. The rate of formation of complex upon the mixing of Hp with Hb did not linearly increase with Hb concentration but became relatively slower. Comparison of

the initial rates indicated that the rate constant decreased 5-fold as the Hb concentration was increased 32-fold.

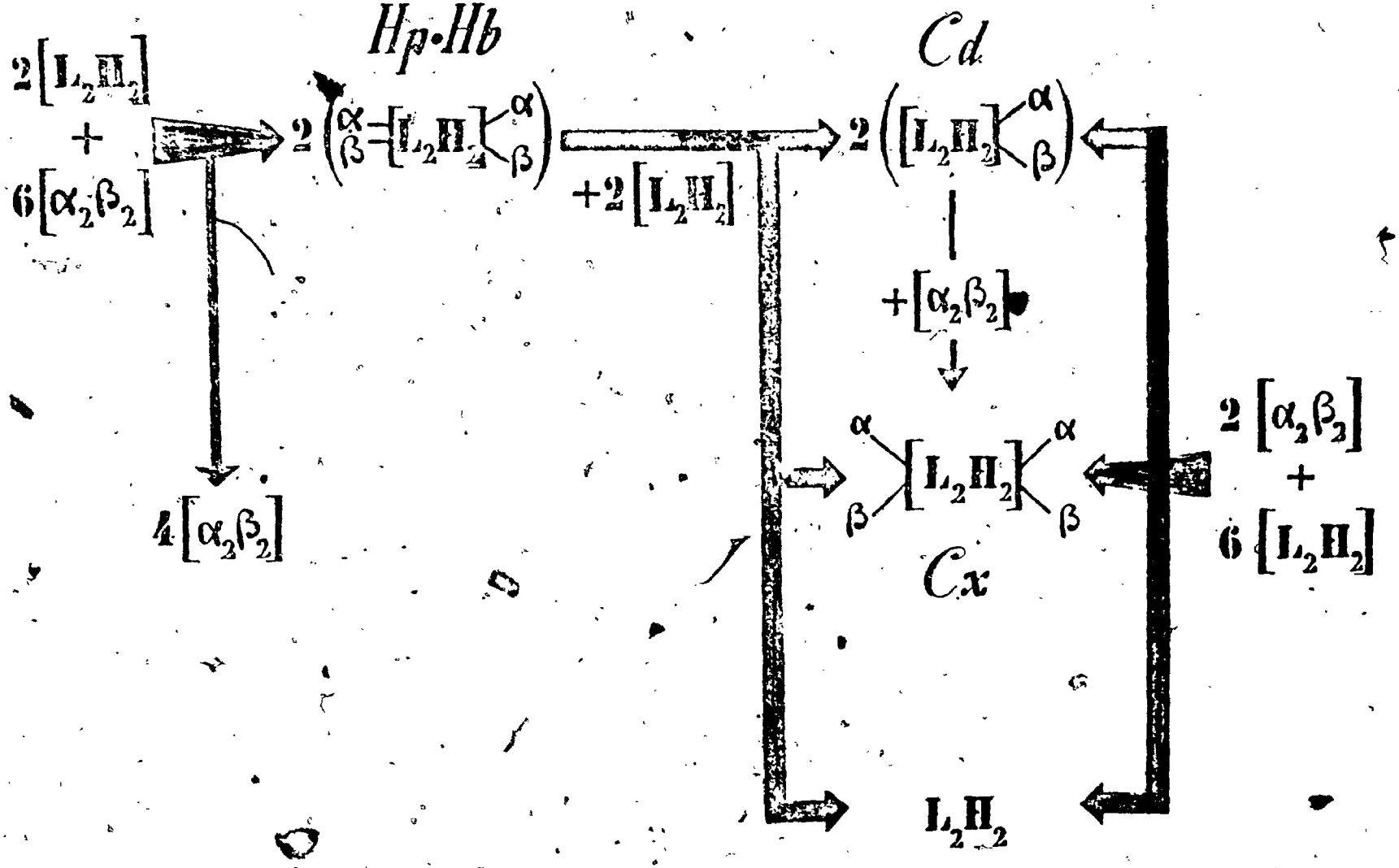
Boyd, Smith and Andrews (1971) have discredited the involvement of isolated monomers by showing that although human and mouse hemoglobins hybridize satisfactorily, bound human α subunits on haptoglobin are replaced by mouse hemoglobin dimer rather than mouse β being added to the Hp α complex to form HpHb.

Investigations of the complex formed by haptoglobin with subsaturating amounts of hemoglobin have further implicated the involvement of dimer. Laurell and Gronvall (1962) speculated that the three components in these preparations as seen by electrophoresis were Hp, HpHb, and Hp bound to one half molecule of hemoglobin. The sedimentation coefficient of this material was intermediate between that of Hp and saturated complex (Hamaguchi, 1966). The material disappeared upon the addition of sufficient Hb to reach or exceed a 1:1 molar ratio, and did not reappear when Hp was added to this saturated complex (Hamaguchi and Sasazuki, 1967). Hamaguchi (1967) found a molecular weight and heme content consistent with the half saturation theory. Ogawa, Kagiyama, and Kawamura (1968) confirmed the molecular weight as 135,000 and suggested that the Hb component was an $\alpha\beta$ dimer rather than either α_2 or β_2.

Immunological proof of this was soon to follow. The intermediate complex bound both α- and β-specific antibodies (Kagiyama, Ogawa, and Kawamura, 1968).

The existence of more than one type of saturated HbHp complex has been suggested. Brunori et al (1968) have pointed out that the shape of the redox equilibrium curve of Hb changes with the molar ratio of Hb to Hp. They report the formation of one type of complex with the molar ratio of Hp:Hb being 2:1 but two types with a molar ratio of 1:1. Waks et al (1969) have investigated this further and have proposed the reaction scheme shown in Figure 10. With excess Hb, Hp forms one type of complex, designated Hp·Hb. With excess Hp, two types of complex are formed, one designated C_x which is saturated with Hb, and the other designated C_d which is half-saturated. The complex C_x is stable to the addition of Hp while Hb·Hp is not so that C_x and C_d result from its exposure to Hp. This system requires the presence of strong and less strong bonds between Hb and Hp, as indicated by diagonal and horizontal lines in the diagram, which must in turn involve thermodynamically more and less favourable modes of association. Thus the rearrangement of Hb·Hp to C_x would be predicted but is not observed. Also, the less strongly bonded dimer might also be expected to show exchange with unbound hemoglobin dimers but Bunn (1967) found none. However it is difficult to

Figure 10: A proposed mechanism of binding between hemoglobin and haptoglobin (Waks et al, 1969). Addition of excess hemoglobin to haptoglobin leads to the saturated complex Hp·Hb, which has two Hb dimers bound to Hp in different fashions. Exposure of this to Hp results in loss of one dimer to form C_d, and C_x when both dimers become bound in the stable manner. Once formed, the stable bond shown by diagonal lines is not altered by exposure either to Hb or Hp. The less stable bond shown by horizontal lines in Hp·Hb is not necessarily an intermediate because C_d and C_x were formed directly by mixing with Hp in excess.


Hp·Hb
Cd
2[L2H2]
+
6[α2β2]
+2[L2H2]
+[α2β2]
4[α2β2]
Cx
2[α2β2]
+
6[L2H2]
L2H2

discount the different proton release curves and inhibition of peroxidase activity kinetics seen by Waks et al.

Different intermediate and saturated complexes of Hp 2-1 and Hp 2-2 with Hb have been detected spectrophotometrically by Pavlicek and Jaenicke (1971). A twofold difference in the number of histidine residues buried when Hb is added to Hp compared to the reverse order of addition has led them to suggest two types of association, one involving essentially tetrameric Hb on the Hp surface and the other involving two Hb dimers bonded to Hp more independently of each other.

Clarification of this subject will require much further work and may only be resolved by X-ray analysis.

In view of the above data and the fact that the interaction of isolated α subunits with free and half-saturated Hp was found to be indistinguishable, Nagel and Gibson (1971) have suggested that Hp is bivalent towards Hb dimers with each site being independent and noninteracting. The reaction of hemoglobin with haptoglobin can therefore be written as:

$$\tfrac{1}{2}\,Hp + {}^{\alpha}\alpha\beta \longrightarrow \tfrac{1}{2}\,Hp\alpha\beta$$

HAPTOGLOBIN HEMOGLOBIN-SUBUNIT COMPLEX

The interaction of the Hb H (β_4) with haptoglobin was reported by Nagel and Ranney (1964) to be, at most, minimal. Only slight fluorescence quenching of Hp by isolated β^{PMB} subunits, and no Hpβ complex could be

demonstrated electrophoretically by Nagel and Gibson (1967). Chiancone, Alfsen et al (1968) found that the affinity of β subunits for Hp was so low that no estimate of the number which could be bound was feasible. This inability of Hp to bind β subunits, however, could be dramatically reversed by the addition of α subunits. Comparison of the interaction of Hp with Hb A ($\alpha_2\beta_2$), Hb H, and isolated α and β subunits suggested that Hp reacts primarily with α subunits and only secondarily with β subunits in the formation of hemoglobin haptoglobin complex. Hp-α subunit studies have therefore warranted more attention. They have also involved more contradiction.

The first paper that involved studies of Hp with isolated α subunits was that of Nagel and Ranney (1964). On the basis of electrophoresis patterns, they suggested that α, like β subunits, could not form complex with Hp and ascribed this failure to interact to probable conformational differences compared with those combined with β subunits in a dimeric or tetrameric form.

Chiancone, Wittenberg et al (1966) reported the detection of Hp α subunit complex by sedimentation velocity experiments in the analytical ultracentrifuge but the study was marred by the formation of a "red precipitate". No estimate of S value, molecular weight, or the number of subunits bound per mole of Hp was made.

The fluorescence quenching measurements of Nagel and Gibson (1967) indicated an equilibrium of the type

Hp + α ⇌ Hpα, and that once the α subunits had bound, rapid binding of β could follow: Hpα + β ⇌ Hp αβ. Even in the presence of a large excess, Hp was not saturated by α^{PMB}, that is, it did not bind four monomers. The existence of two independent binding sites was proposed. Limited binding was observed by electrophoresis. These authors also found no difference in affinity of α^{SH} and α^{PMB} for haptoglobin. Therefore all studies using either form are directly comparable.

Conflicting evidence was later presented by Chiancone, Alfsen et al (1968). Determination of the sedimentation coefficient of Hp complex in the ultracentrifuge with that of HpHb as reference implied that saturation with four α monomers was asymptotically approached in the presence of only slight excess. Electrophoretic separation of mixtures of Hp added to either α or β subunits resulted in three bands to which were assigned the identities of free Hp, free subunit, and Hp-subunit complex. Less of the third was found in the β case. Static fluorescence graphs of quenching versus molar ratio heme to Hp were used to prove that four α subunits bound to Hp. No estimate of the number of β bound could be made due to low affinity. More consistent with previous work was the apparent dependence of Hpα S-value on protein concentration thus confirming the involvement of an equilibrium process of association.

The most recent contribution to this subject is that of Nagel and Gibson (1971). They found that the degree of fluorescence quenching of Hp by α subunits was roughly half that produced by hemoglobin. This suggested that two rather than four sites specific for α are present in haptoglobin. β subunits did not significantly interact with Hp, but in the presence of α, they bound very rapidly and strongly to form a product indistinguishable from HpHb. Thus the binding of α was said to allosterically induce the formation of a highly specific β site. The proximity of these sites could not be determined but two mechanisms of αβ dimer binding to Hp were proposed. Upon binding to the α site, the dimer could either undergo some unknown rearrangement without actual dissociation followed by binding of the β subunit, or the dimer could dissociate due to α subunit conformational changes resulting from its interaction with Hp, and then the β subunit would be free to seek out and attach to the β site. The likelihood of the latter is slight vis-à-vis the displacement of Hp-bound α by mouse dimer (Boyd et al., 1971).

The primary purpose of this work was to investigate further the Hp-α subunit interaction with a view to the resolution of the aforementioned uncertainties. To approach this subject, the biochemical techniques of sedimentation velocity in the analytical ultracentrifuge,

gel exclusion chromatography, and intramolecular cross-linking were considered most applicable.

THE CROSS-LINKING OF PROTEINS

The covalent linkage of polypeptide chains constitutes the single chemical method of determining conformational distances in biological molecules. Naturally occuring links such as disulfide bridges and, to a lesser extent, carbohydrate as well as ester bridges (joining aspartic or glutamic residues with serine or threonine), have provided the means of obtaining much structural information. The introduction of artificial cross-links is fast becoming an invaluable tool in the biochemist's hands to probe tertiary and quaternary structure.

Of major importance in studies of this kind is the selection of modification reagent and conditions so as not to disrupt the native state. Extent of disruption is most often monitored by the assay of biological activity. The suitability of many classes of reagents for application to proteins have been considered in these terms. Solubility, target group specificity, and type(s) of groups modified by these classes are summarized in Table 1. Bifunctional reagents, that is, those which can link two amino acid side chains whose separation distance can be spanned by the reagent, are most useful. Parameters such as protein concentration and net charge, the molar ratio of protein sites to reagent, and reagent length can be chosen so as to favour intra- or intermolecular crosslinks.

Table 1: Reagent classes, with properties, that are considered suitable for crosslinking proteins.

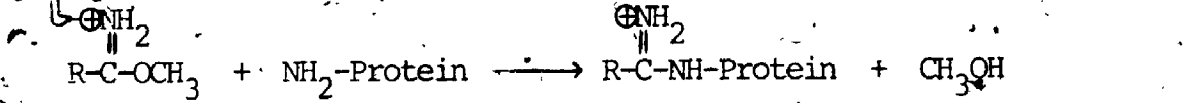
Class	Solubility*	Common Method of Addition	Target Group Specificity	Type(s) of Groups Modified
Maleimide	A	Solid	Very Good	Sulfhydryl
Alkyl Halide	A	Aqueous Solution	Poor	Sulfhydryl, Sulfide, Imidazole, Amino
Aryl Halide	B,C	Acetone Solution	Moderate	Amino, Phenolic; Sulfhydryl, Imidazole
Isocyanate	D	Varied	Good	Primarily Amino
Dialdehyde	C,D	Aq.-Organic Mixture	Poor	Primarily Sulfhydryl and Amino
Imidoester	A	Solid	Very Good	Amino

* Solubility A: Water soluble
B: Insoluble in water
C: Soluble in Aqueous-Organic Mixture
D: Varied according to parent structure

Each of these products have distinct but divergent advantages. An extensive review of the theory and techniques of protein modification is presented in Methods in Enzymology, Vol. XI. The bifunctional reagent literature per se has been reviewed by Alexander et al (1952), by Wold (1967), and by Fasold et al (1971).

Imidoesters and Proteins

The reaction of imidoesters with protein amino groups can be written as follows:

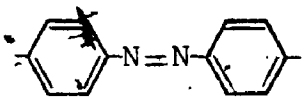


(Hughes, Saroff, and Carney, 1949)

Imidoesters possess many characteristics which make them very suitable for cross-linking studies. First, they are water soluble. Organic solvent reaction mixtures can therefore be avoided. No charge alteration is involved as the amidine formed is less basic than guanidine but has a somewhat higher pK_a than that of an amine (Hunter and Ludwig, 1962; Wofsy and Singer, 1963). The only process that competes with amino amidination is that of reagent hydrolysis.

Extensive or complete amidination of proteins such as bovine serum albumin (BSA) and several antibodies resulted in little if any change in three-dimensional structure as inferred by $[\alpha]_D$, A_{280}, and $S_{20,w}$ measurements. (Wofsy and Singer, 1963).

For solubility reasons, the imidoesters used to

date have not involved the azobenzene structure,  , which confers the advantage of being easily cleaved by dithionite, but rather have been derivatives of straight chain organic acids. However, the amine function can be regenerated by treatment with ammonia-ammonium acetate buffer pH 11.3. This method was developed and applied by Ludwig and Byrne (1962) to amidinated insulin. High voltage electrophoresis of the trypsin digest of this doubly treated protein yielded a peptide map indistinguishable from that of unreacted insulin. The ease of preparation of imidoesters by the condensation of a nitrile and an alcohol in the presence of anhydrous hydrogen chloride,

$$RCN + R'OH + HCl \xrightarrow{\text{Anhydrous}} RC(=NH\cdot HCl)(OR')$$

allows the synthesis of forms tailored to the particular problem at hand. The R groups size, shape, and charge can be varied. The R group can also be coloured, fluorescent, radioactive, or contain a heavy metal atom for X-ray crystallographic studies. In addition, dinitriles can be used to synthesize diimidoesters, that is, bifunctional reagents.

The synthesis of a number of bifunctional imidoesters has been described by Dutton, Adams and Singer (1966). The three examples which have been used to cross-link proteins are diethyl malonimidate (DEM), dimethyl

suberimidate (DMS), and dimethyl adipimidate (DMA) (all as the dihydrochloride) structures are shown in Figure 11. The introduction of a cross-link by protein reaction with DMA is presented in Figure 12 as is modification with the monofunctional imidoester, ethyl acetimidate.

The antigenic sites of BSA and γ-globulin were not lost by extensive reaction with DEM, nor was the ability to bind hapten by anti-DNP antibodies affected by amidination (Dutton et al, 1966). This eliminated the possibility of amino group involvement in the antigen-antibody site. The capability for intermolecular cross-linking was dramatically demonstrated by the formation of a solid gel by the reaction of DEM with a 20% solution of BSA.

A comprehensive study by Hartman and Wold (1967) involved the production of a hyperactive form of bovine pancreatic ribonuclease A upon its exposure to DMA. Analysis of tryptic peptides indicated that crosslinks had been formed between LYS_7 and LYS_{37} as well as LYS_{31} and LYS_{37}. Therefore the maximum distance that could separate these residues in the solvated protein was 8.6 Å, the length of the reagent. This was consistent with the structure of the protein as determined by X-ray crystallography.

The technique of SDS gel electrophoresis as a way of monitoring the degree of crosslinking and molecular

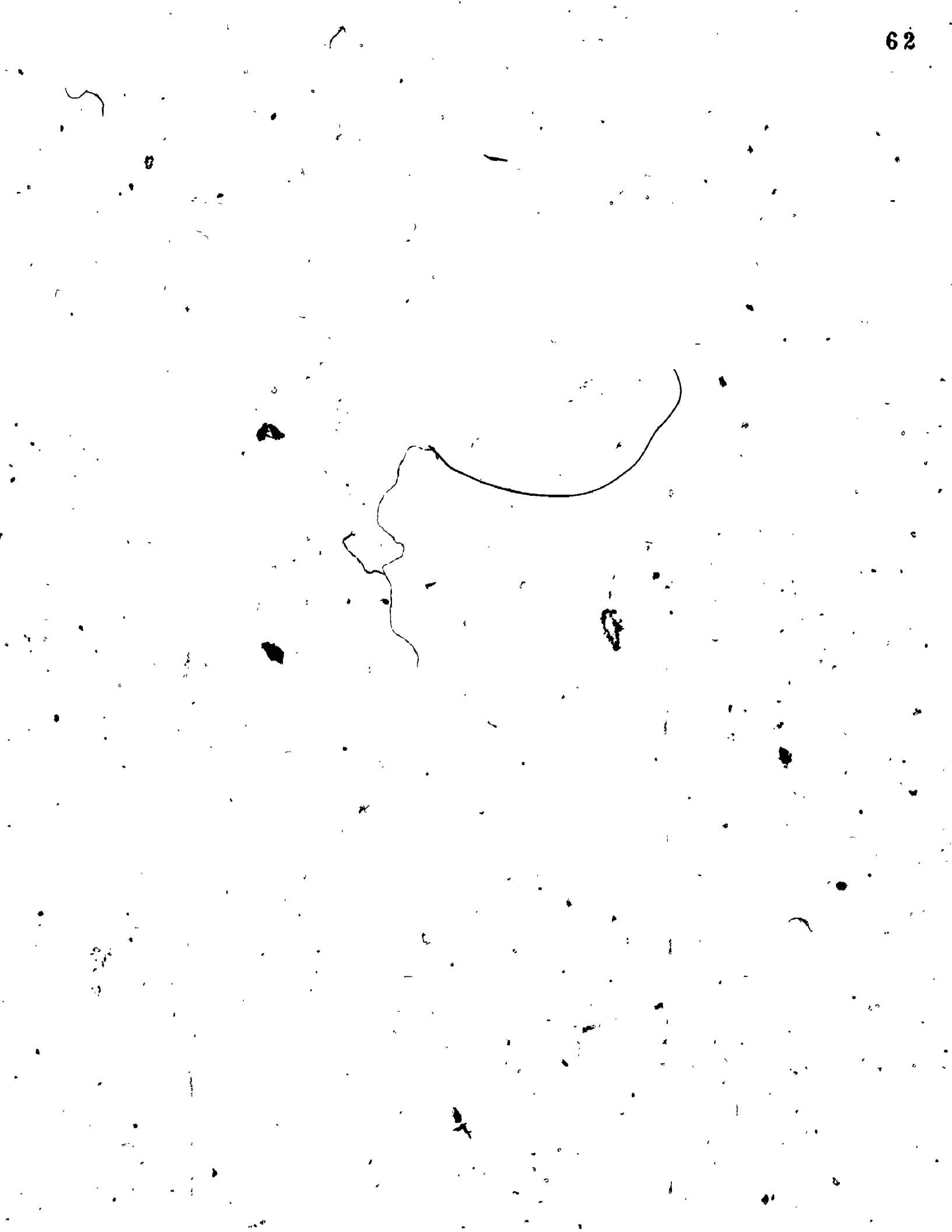
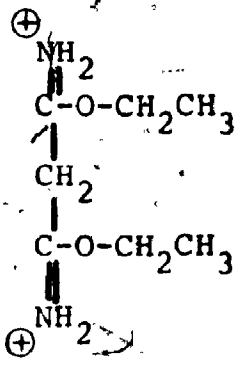
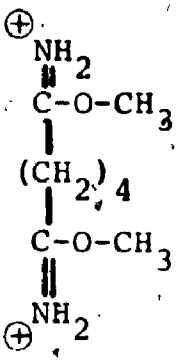


Figure 11: Several bifunctional protein reagents.

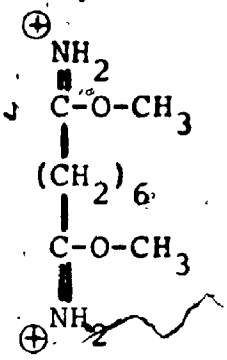
Figure 12: Ethyl acetimidate and dimethyl adipimidate reaction with protein.



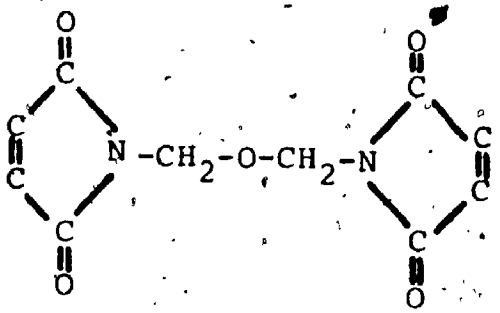
Diethyl Malonimidate (DEM)



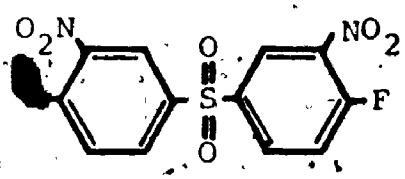
Dimethyl Adipimidate (DMA)



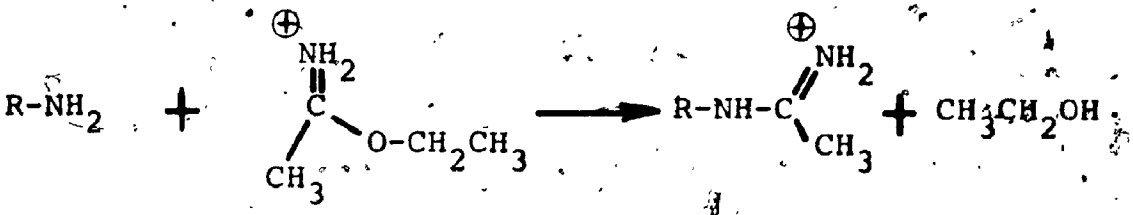
Dimethyl Suberimidate (DMS)



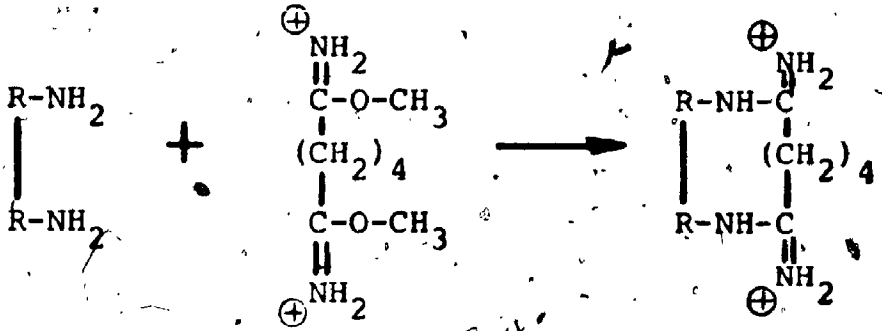
bis(Maleimidomethyl)Ether



p,p'-Difluoro-m,m'-dinitrodiphenylsulfone (FNPS)



Ethyl Acetimidate



Dimethyl Adipimidate

weight of the products has been applied by Davies and Stark (1970) in their studies of several oligomeric proteins reacted with DMS. Only slight deviation from the linear relationship between Log molecular weight and mobility, so beautifully demonstrated by Weber and Osborn (1969), was noted. There was a slight error in the apparent molecular weights of the crosslinked species in the direction of higher values and this was attributed to reduced binding of SDS and/or to increased molecular weight due to incorporation of reagent. In all cases, the number of major bands was equal to the number of protein oligomers. In support of this, Lockhart (in press) has found that four major bands are produced when tetrameric hemoglobin is reacted with DMA.

Pitt-Rivers and Impiombato (1968) have reported that non-reduced proteins bind much less SDS than their reduced forms, and this would imply serious errors would be encountered in the application of the SDS method of MW determination to disulfide or artificially crosslinked proteins. Fish, Reynolds and Tanford (1970) have voiced this opinion (without experimental evidence) but the work of Davies and Stark (1970) tends to establish the validity of the technique even in these cases. Further evidence of Dunker and Rueckert (1969) involving bovine serum albumin whose tertiary structure is maintained by seventeen disulfide bridges has shown an experimental error of only six per cent in the non-reduced form, compared with two

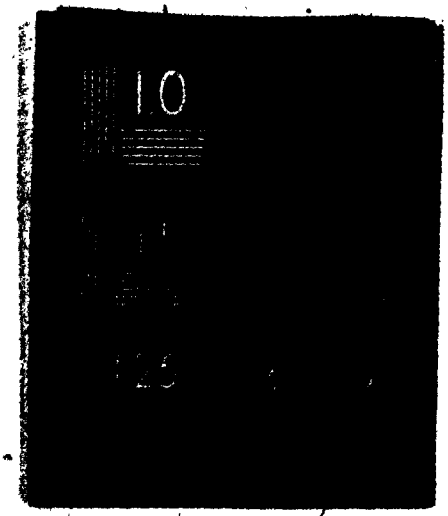
spaced in the solvated and crystalline forms of the molecule. The concommitant loss of heme-heme interaction with this modification which should prevent movement of the $\alpha\beta$ dimer subunits with respect to one another has lent support to the proposal by Perutz et al (1968 a, b) that $\alpha\beta$ rather than $\alpha_2\beta_2$ is the allosterically functional unit of hemoglobin.

Lockhart and Smith (in press) have extensively investigated the use of imidoesters to modify hemoglobin, haptoglobin, and their complex. Preliminary work with the monofunctional reagent ethyl acetimidate implied the promise of this particular approach to the conformational study of the Hb-Hp system. Amidination was found to be complete with no evidence of non-lysine modification. Electrophoretic mobility below pH 9 was identical to that of unreacted Hb. The participation of the N-terminal VAL and LYS 127 of the α subunit in the Bohr effect (Perutz, 1970b) precluded its loss while some heme-heme interaction was retained. Most important however, the ability to bind to Hp was unchanged.

Hemoglobin was selected as the model system to establish optimum conditions for intramolecular cross-linking with the bifunctional dimethyl adipimidate. Reaction was essentially complete after one hour at room temperature and pH 9.5 in 0.1 M sodium borate. Reagent uptake was near maximum with a ten-fold ratio of DMA to

25

OF/DE



per cent in the reduced form. Thus the method is clearly useful in this manner as well as in determining the number of protein subunits.

Crosslinking Studies with Hemoglobin

The bifunctional maleimide derivative bis(N-maleimidomethyl)ether (BME) was reported by Simon and Konigsberg (1966) to react specifically with the well-exposed β-93 sulfhydryls. The structure of the reagent is given in figure 12. Two moles of reagent were incorporated per mole of Hb and no interchain crosslinks formed. Arndt et al (1971) have found that most of the BME forms a bridge with histidine β-97. Both the strength and extent of interaction of Hb-BME_2 with haptoglobin are reduced compared to that of unreacted Hb and displacement readily occurs (Bunn, 1967). Both a decreased ability to dimerize and conformational constraints have been invoked as reasons for the modified properties observed. The fact that the molecule is locked in the liganded conformation, independent of the presence of ligand (Simon et al, 1967) has allowed Perutz (1970 a) to discover the molecular motions involved in the allosteric mechanism of ligand binding of hemoglobin.

The crosslinking of the amino termini of the two α subunits in the hemoglobin tetramer with p,p'-difluoro-m,m'-dinitrodiphenylsulfone (FNPS; structure in Figure 12) by Macleod and Hill (1970) has shown that these groups are similarly

spaced in the solvated and crystalline forms of the molecule. The concommitant loss of heme-heme interaction with this modification which should prevent movement of the $\alpha\beta$ dimer subunits with respect to one another has lent support to the proposal by Perutz et al (1968 a, b) that $\alpha\beta$ rather than $\alpha_2\beta_2$ is the allosterically functional unit of hemoglobin.

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lysyl residues. The third variable, protein concentration, to be chosen to favour the linking of amino groups within chains and between chains within molecules rather than between chains between molecules was also investigated. A hemoglobin concentration of 0.2 per cent yielded no polymer and thus 0.1 per cent was recommended and used.

Physical properties of hemoglobin such as sedimentation coefficient and Stokes radius were essentially unaffected by crosslinking in these conditions, while ability to bind Hp was reduced to about one third that of unreacted Hb probably due to crosslink-induced conformational constraints. SDS gel electrophoresis indicated the presence of four major components which were interpreted as monomer, dimer, trimer, and tetramer on the basis of their mobility. The dimer consisted of two similar components, both of which seemed capable of forming complex with Hp. When the crosslinking reaction was performed at 0.001 per cent only one dimer was seen. This was tentatively assigned the $\alpha_1\beta_1$ identity.

Isolation of the four Hb-DMA components in non-denaturing conditions was not possible and thus the binding of each to Hp could not be clearly defined. However the complex formed by incubating haptoglobin with Hb-DMA was prepared by column chromatography and subjected to SDS gel electrophoresis. Both dimers as well as the other three components bound to some extent but were not incapable

of stable complex formation. Material dissociable to monomer seemed to interact with Hp to the greatest extent. This was seen as indicating steric interference of binding of the crosslinked components rather than as evidence against the dimer hypothesis of hemoglobin interaction with haptoglobin. Fractionation of Hb-DMA in SDS or other denaturants on a preparative scale was unsuccessful. This prevented the determination of subunit content and the position of cross-link(s) in the DMA-maintained dimer , trimer and tetramer fractions.

DMA-treated HpHb complex consisted of a more heterogeneous assortments of components than that of the more simple hemoglobin case due to the greater number of permutations of chain attachment. However a band of mobility corresponding to the αβ dimer was evident in SDS gel electrophoresis thus lending weight to the argument that α and β subunits bound to haptoglobin are in close proximity.

This work of Lockhart and Smith serves as an essential background to the crosslinking studies of the α subunit of hemoglobin with DMA to be presented in this thesis.

It should be noted that the term intramolecular cross-linking is here used to refer to the introduction of a chemical bridge within a single molecular entity, whether or not this entity is dissociable to constituent parts. Thus Hb tetramer as well as α subunit monomer can be intramolecularly crosslinked.

MATERIALS AND METHODS

MATERIALS

Chemicals

Reagent grade p-chloromercuribenzoate (PCMB) and -methyl-D-mannoside were obtained from Sigma Chemical Company. Amino Acid Calibration Mixture was purchased from Beckman Instruments Inc. and Isoelectric Focussing Carrier Ampholytes from LKB-Produkter, Sweden. All were stored cold and dark.

Ethyl acetimidate HCl and dimethyl adipimidate dihydrochloride were obtained from Pierce Chemical Co. and stored in a dessicator over Drierite.

All other chemicals were of reagent grade.

Resins and Gels

Sephadex gels of various G-numbers, DEAE-Sephadex, and Concanavalin A-Sepharose 4B were obtained from Pharmacia Fine Chemicals (Canada) Ltd.

DEAE-cellulose was obtained as Whatman DE-52 from Mandel Scientific Co. Ltd. and as Cellex-D from Bio-Rad Laboratories. Bio-Rad was also the source of carboxymethyl-

cellulose, marketed as Cellex-CM.

Commercially Prepared Proteins

Sperm whale myoglobin (crystalline, salt free, lyophilized) was obtained from Mann Research Laboratories. Sigma Chemical Company supplied horse heart cytochrome C (crystalline, Type III, lyophilized).

Membranes and Filters

Diaflow ultrafiltration membranes were manufactured by the Amicon Corporation, and Millipore membranes by Millipore Ltd. Whatman #1 and Reeve Angel fast flow filter papers were used. Dialysis tubing was obtained from the A.H. Thomas Co.

METHODS

Amino Acid Analysis

Protein samples were hydrolyzed in 6.0 N HCl in evacuated sealed tubes for 18 hours at 108°C, passed through Whatman #1 filter paper, dried using a Buchler Flash-Evaporator, and dissolved in pH 2.2 citrate buffer. The hydrolyzates were analyzed on a Beckman Auto-Programmed 120C amino acid analyzer using the technique and conditions outlined in the instrument instruction manual. Residues were identified and quantified by comparison with peak position and area obtained from a Beckman calibration mixture analyzed immediately before and/or after the sample

of interest.

Spectroscopy

Gel filtration column eluants were monitored by a Beckman DB or DB-G spectrophotometer. Precise absorbances were obtained with the Gilford Model 2400 spectrophotmeter. Visible and ultraviolet spectra were studied using the Cary Recording Spectrophotometer, models 14 and 15.

Millipore Filtration

Protein solutions contained in B-D plastipak syringes were forced through a Swinnex-25 or Swinney adapter fitted with a 0.45 μ pore size filter. Filtrates were collected in culture or test tubes of appropriate capacity.

Lyophilization

After removal of non-volatile salts by exhaustive dialysis, samples were frozen in a dry ice-acetone bath and lyophilized by a Virtis Model 10-147 MR-BA freeze drier at a temperature of -50°C, 40 microns pressure for 24 hours.

Ultrafiltration

Haptoglobin was concentrated in Amicon 50, 400 and 8MC cells with XM 50 membranes under 50 pounds nitrogen pressure. Ultrafiltration of hemoglobin subunits was in

general avoided as non-reversible adsorption onto UM-10 membranes was found to occur at both 5 and 20°C with various stirring rates.

Polyacrylamide Gel Electrophoresis (PAGE)

Protein mixtures were investigated in acrylamide gel matrix according to the method of Davis (1964) involving the TRIS-glycine buffer (pH 8.9) and with a slightly modified medium pH range system based on that of Williams and Reisfield (1964) consisting of a TRIS-diethylbarbituric acid buffer with separation gel pH of 7.8 and stacking gel pH of 5.5. To avoid the photocatalyzed production of hemoglobin artefacts reported by Peacock et al (1970) sample gels were not used. Instead, samples were layered onto the gel surface in 20% sucrose. In some cases, ionic rather than pH discontinuity was employed with stacking gel omitted. Samples were electrophoresed in 6 mm inner diameter glass tubes and also in a rectangular cell as part of a slab gel system manufactured by ORTEC Inc.

A study of haptoglobin microheterogeneity by PAGE involved the TRIS-glycine pH 8.9 electrode buffer of Ornstein and Davis (1964) combined with the TRIS-diethylbarbituric acid pH 7.8 gel system similar to that of Williams and Reisfield (1964). Ten microliter samples made sample and stacking gels unnecessary.

Preparative scale gel electrophoresis was performed

with the Canalco Prep-Disc apparatus with accompanying Beckman Accu-Flow pump and Model 132 Fraction Collector according to the recommendations of the manufacturer and using the pH 8.9 PAGE system of Ornstein (1964) and Davis (1964).

The method of Weber and Osborn (1969) was followed for gel electrophoresis in sodium dodecyl sulfate.

To avoid possible Joule heat denaturation, all PAGE experiments involving hemoglobin subunits, in the absence of SDS, were done with the samples prepared in ice-cooled culture tubes and run with the samples and gels fully immersed in the lower reservoir tank filled with precooled stirred buffer also surrounded by an ice-water bath.

Gel Band Densitometry

Following staining with Amido Black, Coomassie Brilliant Blue, or bromophenol blue and appropriate destaining, acrylamide gels were scanned with the Gilford Model 2400 spectrophotometer fitted with a Linear Transport assembly.

When integration to determine relative amounts of components was performed, the bands stained with Coomassie Blue always contained less than the 15 ug protein maximum recommended by Weber et al (1972) as the upper limit of the range inwhich stain uptake is linearly proportional to protein present.

Thin Layer Gel Filtration (TLG)

Methodology employed was that recommended by Pharmacia

accompanying their TLG apparatus. In particular, 0.8 mm G-75 and G-150 Sephadex gel layers on 20 X 40 cm glass plates equilibrated at 4-6°C at an angle of 25° were used. Protein distributions resulting from chromatography of 5 ul samples were absorbed onto 3 MM Whatman paper and stained in a Coomassie Blue bath and the background appropriately destained.

Analytical Gel Filtration

The technique of analytical gel filtration was carried out as described in the pamphlet published by Pharmacia entitled Gel Filtration in Theory and Practice. A 2.5 X 50 cm Pharmacia column packed with G-150 Superfine gel equilibrated in 0.1 M sodium phosphate - 2 X 10^{-5} M EDTA, pH 7.5 (PO_4-EDTA) buffer was employed. Sample volume was 2.0 ml.

Preparation of Hemoglobin

Human hemoglobin was prepared from recently outdated citrated whole blood obtained from a local hospital blood bank. All centrifugations were performed in a 0-5°C refrigerated Sorvall RC2-B instrument using 500 ml liquid-tight tubes in a three liter capacity rotor. The erythrocytes were separated by centrifugation for 15 minutes at 8000 rpm and then washed five times with six volumes of 0.15 M saline. Hemolysis was induced according to the method of Drabkin (1946) by mixing with an equal volume of

distilled water and 0.4 volumes of toluene followed by repeated inversion for 15 minutes at 5°C in a glass stoppered cylinder. The released hemoglobin was separated from the cell debris and toluene-lipid extract by centrifugation (30 minutes at 8000 rpm) and filtration through Whatman #1 paper. After 24 hour dialysis against 200 volumes of 0.1 M NaCl - 4 X 10^{-5} M EDTA, the resulting hemoglobin solution, whose concentration was approximately 7%, was stored for a maximum of one month after preparation.

Determination of Hemoglobin Concentration

An approximate estimate of hemoglobin concentration was made by applying Beer's Law using monomer millimolar extinction values of 15.0 at 540 nm or 125.0 at 413 nm as quoted in the Merck Index for HbO_2. More precise measurements were obtained after conversion of an aliquot of known volume to the cyanmet derivative with HYCEL reagent, and comparison of the absorbance of several dilutions with a calibration curve prepared with a standard solution also supplied by HYCEL Inc.

Preparation of Hemoglobin α^{SH} and α^{PMB} Subunits

The method used was that of Geraci, Parkhurst and Gibson (1969), with modification.

The sulfhydryl reagent p-chloromercuribenzoate (125 mg) was added to 20 ml of a pH 6.7 solution of 6-8% Hb which was 80 mM in sodium chloride and 4 X 10^{-5} molar in EDTA.

This and all following procedures were performed at 5°C. Sodium phosphate was used exclusively as buffer salt. All buffers except that used in the final Sephadex G-75 step were 10 mM phosphate containing 2 X 10^{-5} M EDTA. After gentle efficient stirring for ten hours, undissolved reagent was removed by Whatman #1 filtration, and the solution was applied to a 4 X 50 cm Sephadex G-25 column pre-equilibrated at pH 8.0. This step removed dissolved reagent, lowered the ionic strength, and adjusted the pH to buffered pH 8.0 conditions. The chemically modified α subunits were then separated by a 3 X 15 cm DEAE-Sephadex column, equilibrated and washed with pH 8.0 buffer.

The pH of the resulting α subunit solution was lowered to 6.6 with 1% acetic acid, and gently stirred in the presence of CM-cellulose for 15 minutes. The α subunit-CM-cellulose was then transferred to a 9.5 cm diameter Buchner funnel and washed with 3 liters of pH 6.6 buffer containing 5 ml of β-mercaptoethanol (βME) to remove the PMB group on the α 104 cysteine residues. The βME was then washed out with 3 liters of pH 6.6 buffer. The resin was then resuspended and packed in a 1 X 15 or 3 X 15 cm column and eluted with 0.1 M phosphate, pH 7.5, containing 2 X 10^{-5} M EDTA (PO_4-EDTA).

The inclusion of an additional step involving chromatography through a 2.5 X 90 cm G-75 Sephadex column yielded pure α^{SH} subunits. The necessity of this step, identity of the contaminant removed, and proof of subunit purity are

presented in the Results section of this thesis.

The preparation of α^{PMB} subunits followed that of α^{SH} subunits detailed above with omission of the steps involving CM-cellulose.

A flow chart is presented in Figure 13.

Determination of Sulfhydryl Groups

Sulfhydryl groups of proteins were titrated with p-chloromercuribenzoate (PCMB) according to the method of Boyer (1954).

A concentrated PCMB solution was prepared by dissolving several crystals of the reagent in PO_4-EDTA which was made slightly alkaline by the addition of sodium hydroxide. A volume of this solution was diluted ten times with PO_4-EDTA and denoted standard PCMB. The concentration of this standard solution was then determined by measurement of the increase in absorbance at 232 nm (corrected for dilution) of 3.00 ml of PO_4-EDTA upon the addition of 30 ul aliquots of the standard solution. A molar extinction coefficient of 1.69×10^4 $M^{-1}cm^{-1}$ was employed.

An accurately known volume of the protein solution to be titrated was placed in a dried cuvet in the Gilford 2400 Spectrophotometer. The blank cuvet contained the same solution. Aliquots of the standard PCMB solution were added to the sample and the absorbance at 250 nm was recorded. The graph of absorbance, corrected for dilution, versus volume of PCMB added was bilinear. See Figure 14.

Figure 13: Flow chart of α subunit preparation.

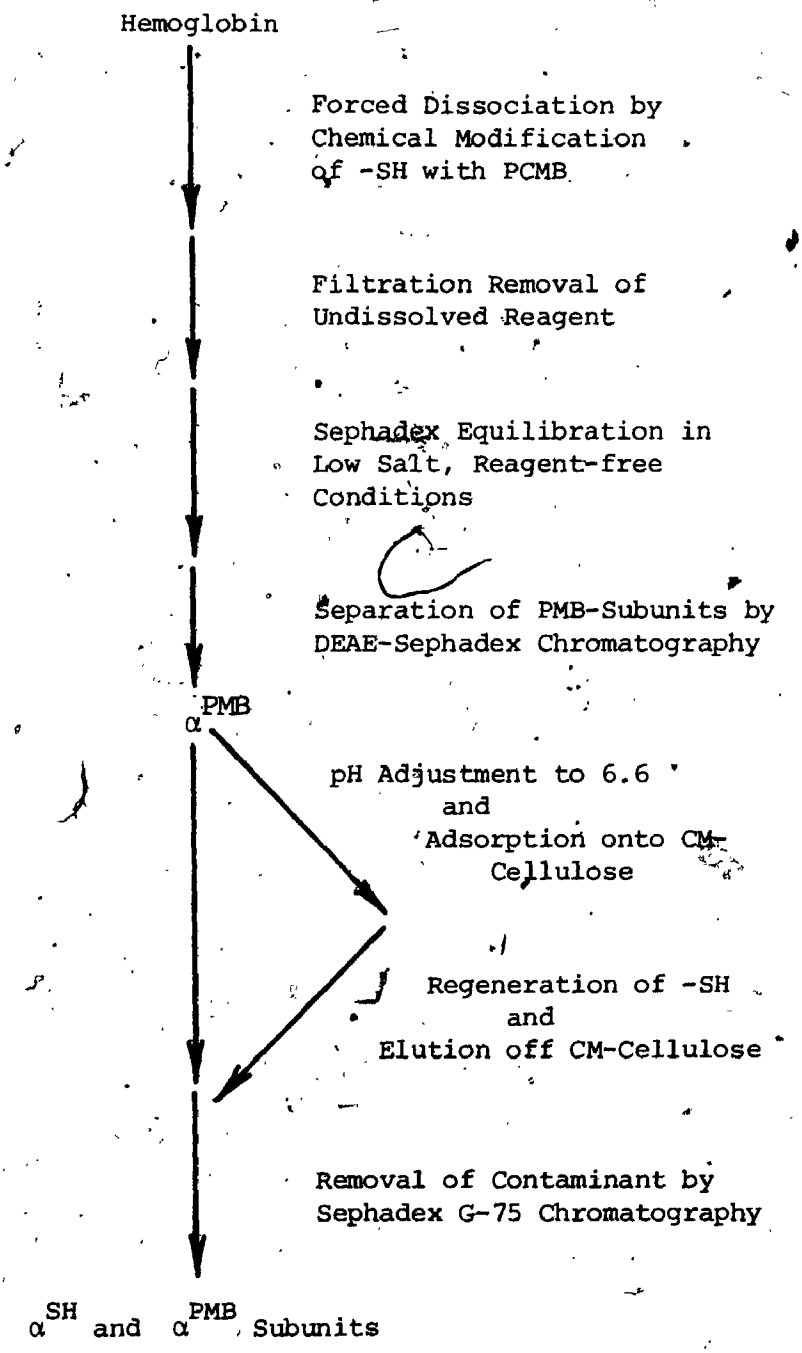
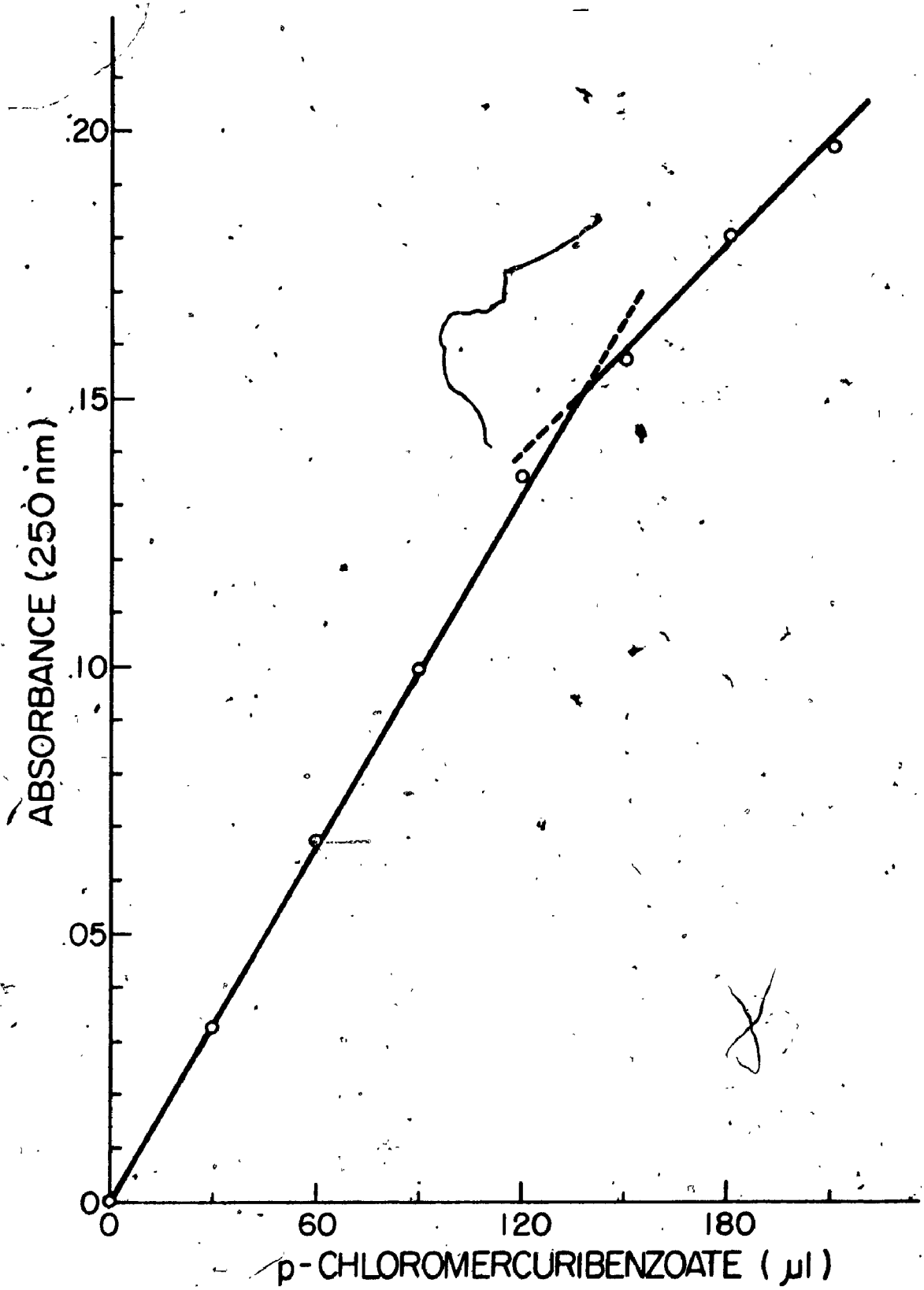

Hemoglobin
Forced Dissociation by Chemical Modification of -SH with PCMB
Filtration Removal of Undissolved Reagent
Sephadex Equilibration in Low Salt, Reagent-free Conditions
Separation of PMB-Subunits by DEAE-Sephadex Chromatography
α PMB
pH Adjustment to 6.6 and Adsorption onto CM-Cellulose
Regeneration of -SH and Elution off CM-Cellulose
Removal of Contaminant by Sephadex G-75 Chromatography
α SH and α PMB Subunits

Figure 14: Sulfhydryl titration of hemoglobin α subunit with p-chloromercuribenzoate.


.20
.15
.10
.05
0
ABSORBANCE (250 nm)
0
60
120
180
p-CHLOROMERCURIBENZOATE (µl)

The initial phase corresponded to mercaptide formation by the reaction of sulfhydryl with PCMB while the final phase represented the addition of excess reagent. The end point of the titration was taken as the point of intersection of these two lines. The number of available sulfhydryls per molecule of protein could therefore be calculated using this end point and the protein concentration which was determined spectrophotometrically.

Determination of Haptoglobin Concentration

Herman-Boussier et al (1960) have reported that a 1mg/ml solution of haptoglobin has a 280 nm absorbance of 1.2. However, a modification of the technique of Lionetti et al was routinely used as it afforded great accuracy and detected haptoglobin solely on the basis of its ability to bind hemoglobin, and was therefore independent of purity.

An aliquot of stock HbO_2 (accurately known concentration) was added to a known volume of haptoglobin solution. Sufficient Hb was used to ensure an excess. Pedersen pipets were employed. A 500 ul portion of the resulting solution was applied by means of a Beckman Manual Sample Injection Valve into a 25 X 0.9 cm Chromatronix column of Sephadex G-100 equilibrated with 0.15 M NaCl. Constant flow rate was maintained with a Milton Roy Mini Pump and the column eluant monitored at 413 nm by a Beckman DB Spectrophotometer equipped with a flow cell. The absorbance was automatically plotted by a Sargent-Welch Model SRLG recorder. A typical

elution profile is shown in Figure 15. The haptoglobin concentration was calculated by the following relationship:

$$[Hp] = \frac{\text{Area of HpHb}}{\text{Area of HpHb} + \text{Area of Hb}_{excess}} \cdot [Hb]_{stock} \cdot \text{Dilution Factor}$$

Preparation of Haptoglobin

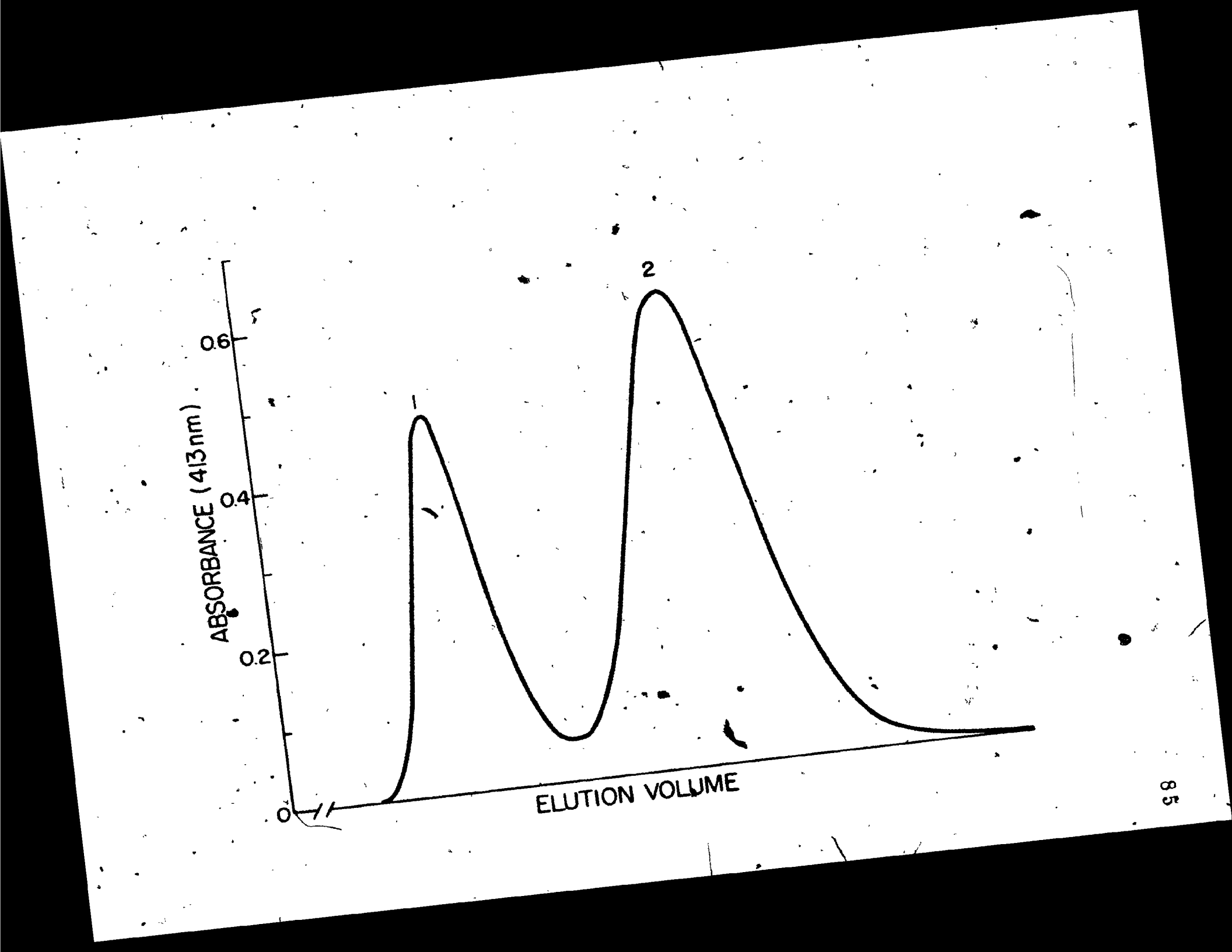
Haptoglobin was prepared from pig blood by a procedure adapted form those of Connell and Shaw (1961), Smith, Edman and Owen (1962), Killander (1964), and Fraser and Smith (1969).

Five gallon quantities of pig blood containing 150 ml of an anticoagulant solution (0.075 M trisodium citrate, 0.038 M citric acid, 0.124 M dextrose) per liter were obtained from an abattoir. After partial erythrocyte removal by settling for 4 hours, the plasma-rich supernatant was removed by siphon and centrifuged (7000 rpm, 15 minutes) to remove all remaining cells. The plasma was desalted by G-25 Sephadex column chromatography (500 ml samples applied to an 8.5 X 60 cm column equilibrated with distilled water) or repeated dialysis against forty gallons of distilled water. A heavy white precipitate appeared and was filtered off through cheesecloth. Three liter portions of deionized plasma, whose conductivity was less than 400 umho, were titrated to pH 4.7 with 0.5 N acetic acid, filtered through fast flow paper to remove a fine white precipitate, and then exposed to 500 g of preswollen Whatman DE-52 equilibrated with 5 mM acetate buffer, pH 4.7.

Figure 15: Determination of haptoglobin concentration by the assay of hemoglobin binding capacity by filtration through a column of Sephadex G-100.

Peak 1: Hemoglobin-Haptoglobin Complex

Peak 2: Excess Hemoglobin


ABSORBANCE (413nm)
0.6
0.4
0.2
0
1
2
ELUTION VOLUME

Common column technique involving elution with a linear salt gradient (0.01 M NaCl to 0.20 M NaCl, in 5 mM acetate pH 4.7) resulted in the profile presented in Figure 16 but also involved a poor yield of haptoglobin. Therefore a batch method was favoured since the time of exposure to these acidic conditions could in this way be greatly decreased. Plasma was added to the DEAE-cellulose, stirred for 10 minutes, filtered in a large Buchner funnel under reduced pressure and washed with approximately 1 liter of acetate buffer until the filtrate had an absorbance at 280 nm of less than 0.1. The resin was then stirred with 1 liter of 0.5 M NaCl in acetate buffer and filtered in a similar manner. The pH was then raised to 7.0 with 0.5 N NaOH. This modification resulted in retention of 75% of the Hb binding capacity present in the deionized plasma.

Salting out with ammonium sulfate according to the nomograph reported by Di Jeso (1968) resulted in minimal loss with the haptoglobin found in the 58 to 70% saturation precipitate.

All steps of the isolation of this semi-pure Hp were performed at 5°C, and monitored with the Hb binding capacity method described above. By these preparative procedures, one gram of semi-pure Hp was obtained from three liters of deionized plasma; this constituted a 60% yield of the Hb binding capacity initially present.

Further purification of Hp in amounts of 3 to 4 grams total protein by G-150 SF and G-200 SF Sephadex gel filtra-

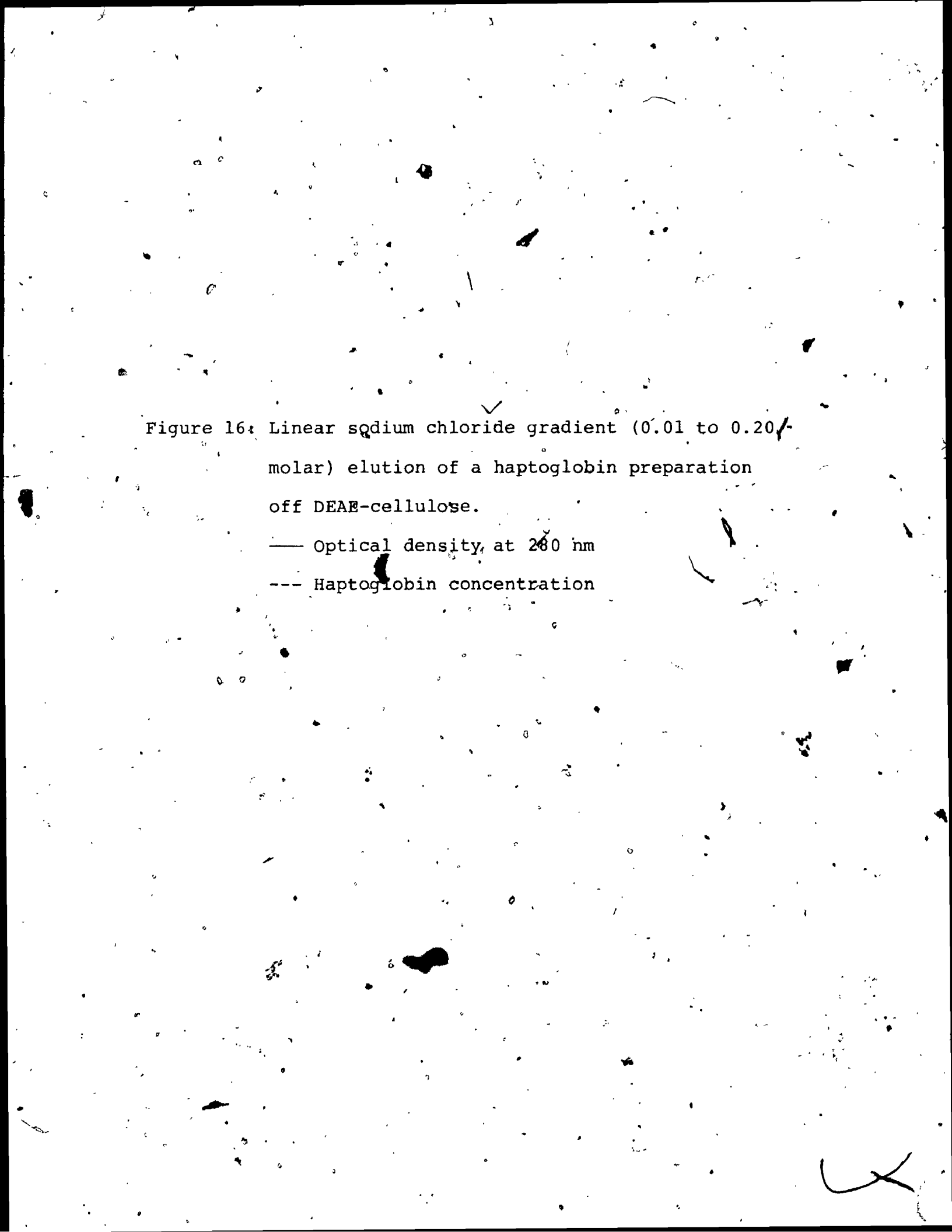


Figure 16: Linear sodium chloride gradient (0.01 to 0.20 molar) elution of a haptoglobin preparation off DEAE-cellulose.

—— Optical density at 280 nm

--- Haptoglobin concentration

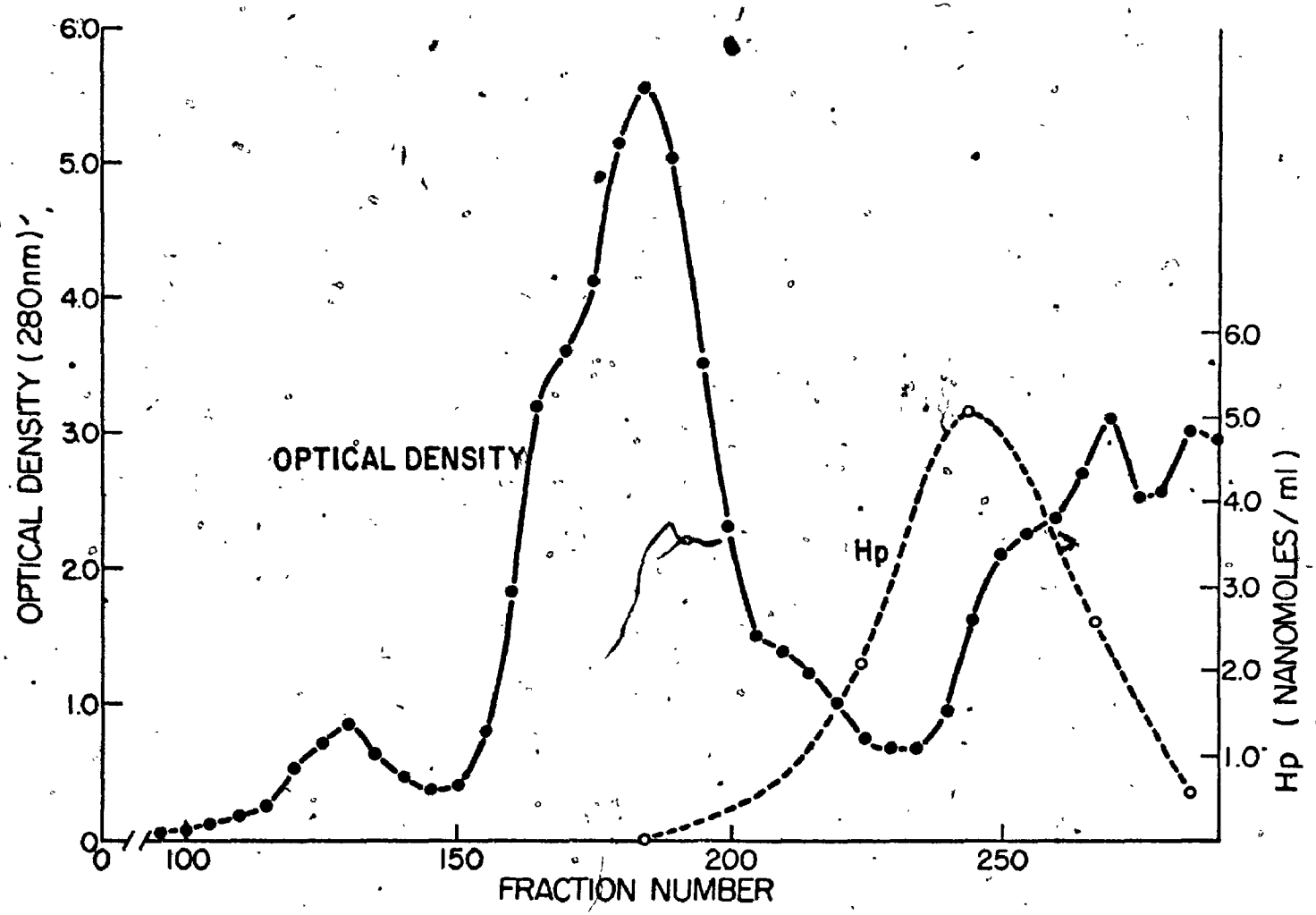
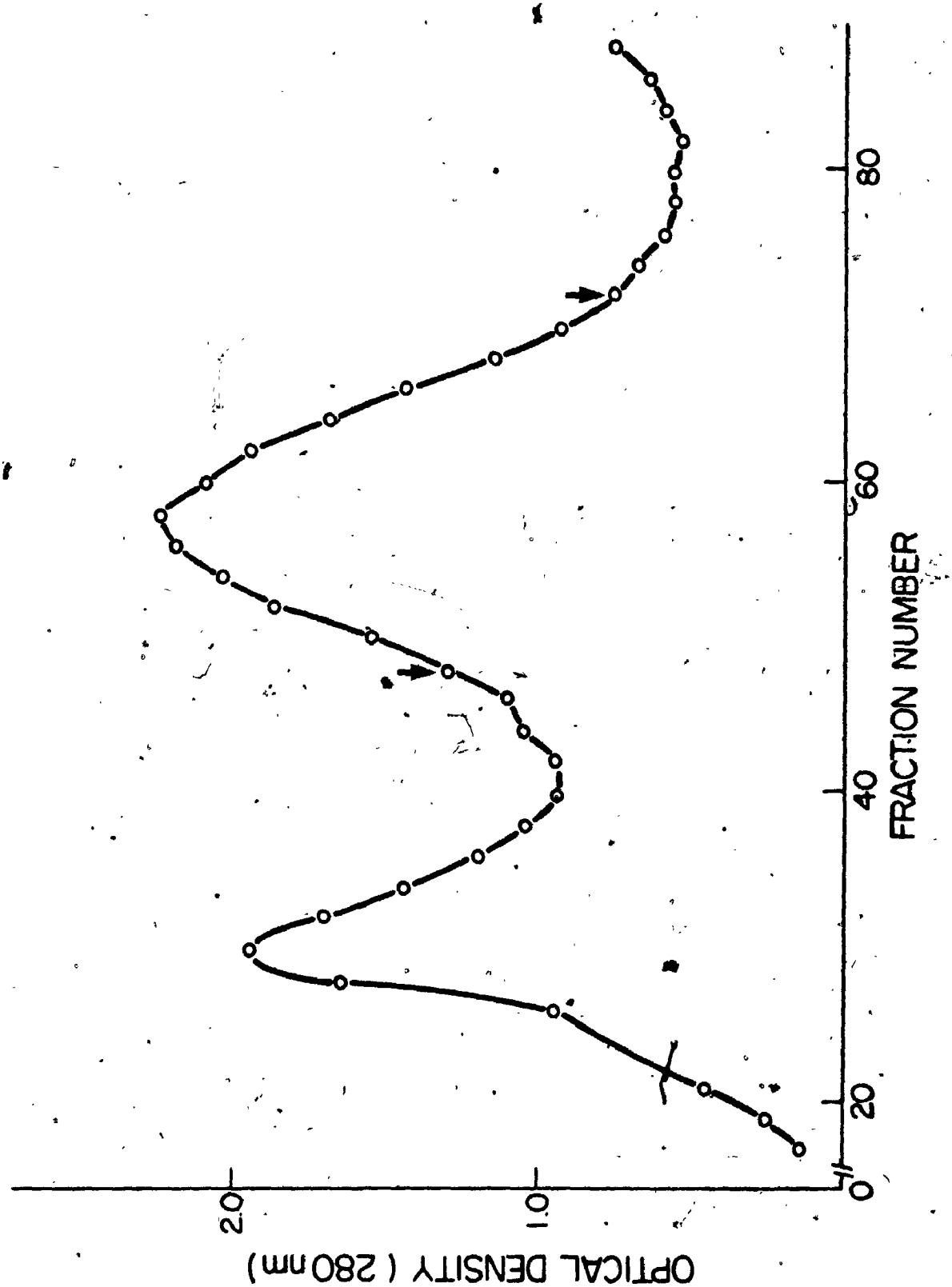

OPTICAL DENSITY (280nm)
OPTICAL DENSITY
Hp
Hp (NANOMOLES / ml)
FRACTION NUMBER
0
1.0
2.0
3.0
4.0
5.0
6.0
100
150
200
250

Figure 17: Elution profile of semi-pure haptoglobin chromatography through a 5 X 100 cm Sephadex G-200 SF column. Fractions pooled lie between and include those indicated by arrows.


OPTICAL DENSITY (280nm)
2.0
1.0
0
20
40
60
80
FRACTION NUMBER

tion in a 5 X 100 cm column equilibrated with 0.15 M NaCl and 0.02% sodium azide yielded similar elution profiles. Figure 17 presents that obtained from chromatography through G-200 SF. Haptoglobin fractions were pooled. Purity was investigated by PAGE and SDS gel electrophoresis, and the material was lyophilized.

Prep-Disc electrophoresis and affinity chromatography involving Concanavalin A-Sepharose 4B were necessary to achieve virtual 100% purity and are discussed in the Results section.

Preparation of Con A-Sepharose 4B

Commercially obtained Concanavalin A covalently linked to Sepharose 4B was prepared for affinity chromatography purification of Hp by combining 100 ml of fresh gel with 65 ml that had been previously exposed to α-D-glucose and regenerated by a treatment method modeled on that of Lloyd (1970) involving a 200 bed volume wash. In the design of the treatment, care was taken to avoid formation of the slightly soluble salt, calcium phosphate. The protocol was as follows:

1. Pack in a 2.5 X 40 cm Pharmacia column.
2. Removal of storage solution containing heavy metal ions: Wash with 400 ml of 0.1 M NaCl in 0.02% sodium azide.
3. Carbohydrate and probable metal ion removal: Wash with 7000 ml of 0.2 M phosphate, pH 6.6. Flow rate

of 100 ml/hour.

4. Removal of phosphate: Wash with 500 ml of 0.1 M NaCl in 0.02% azide.
5. Reactivate binding sites by replacement of metal ions: Wash with 300 ml of 1 mM $CaCl_2$, $MgCl_2$, $MnCl_2$.
6. pH adjustment and return to bacteriostatic conditions: Wash with 500 ml of 0.1 M sodium acetate, pH 6.0 containing 0.02% azide.

This material was stored at 5°C until needed. Immediately prior to use, it was washed with five bed volumes of 0.1 M NaCl.

Affinity Chromatography of Haptoglobin

Preliminary investigations were undertaken to gain insight into the nature of haptoglobin interaction with Concanavalin A immobilized in an agarose matrix as Con A-Sepharose 4B.

The release of haptoglobin bound to the gel was studied as a function of α-D-mannose concentration, by continual washing with 0.1 molar followed by 1.0 molar α-D-glucose, and by a linear glucose gradient. Binding of haptoglobin by the gel was studied by monitoring the Hp content of the effluent from a Con a-Sepharose 4B column to which was applied successive samples of impure haptoglobin. This led to the selection of a batch method as the technique of choice.

To 165 ml of Con A-Sepharose 4B was added 140 ml of solution containing 290 mg of haptoglobin as well as an

undetermined but similar amount of albumin. After incubation for six hours with gentle stirring and for twenty-four hours without, the gel was packed in a 2.5 X 25 cm column and washed with five bed volumes of PO_4-NaCl buffer. The washings contained Hp in amounts barely detectable by the Hb binding capacity method. The gel was then incubated with gentle stirring in 0.5 M glucose for six hours, repacked as a column and the released protein washed out. A second six hour incubation released no further protein based on 280 nm absorbance of the supernatant. PAGE was then used to determine the effectivenesss of haptoglobin adsorption as well as its purity upon elution.

Bacterial Control

0.02% sodium azide was used in procedures involving Hp but strictly avoided in all methods involving hemoglobin or its subunits to avoid heme adsorption (Kellet and Schachman, 1971). Haptoglobin to be stored more than a week was frozen as a solution or lyophilized, while Hb solutions were stored saturated with toluene. Hemoglobin subunits were prepared and stored at 5°C, and with the exception of time-stability studies, were not kept for experimental purposes more than 3 days after preparation. The 2×10^{-5} molar EDTA always present in these subunit solutions might also have had some bacteriocidal effect.

Analytical Ultracentrifugation

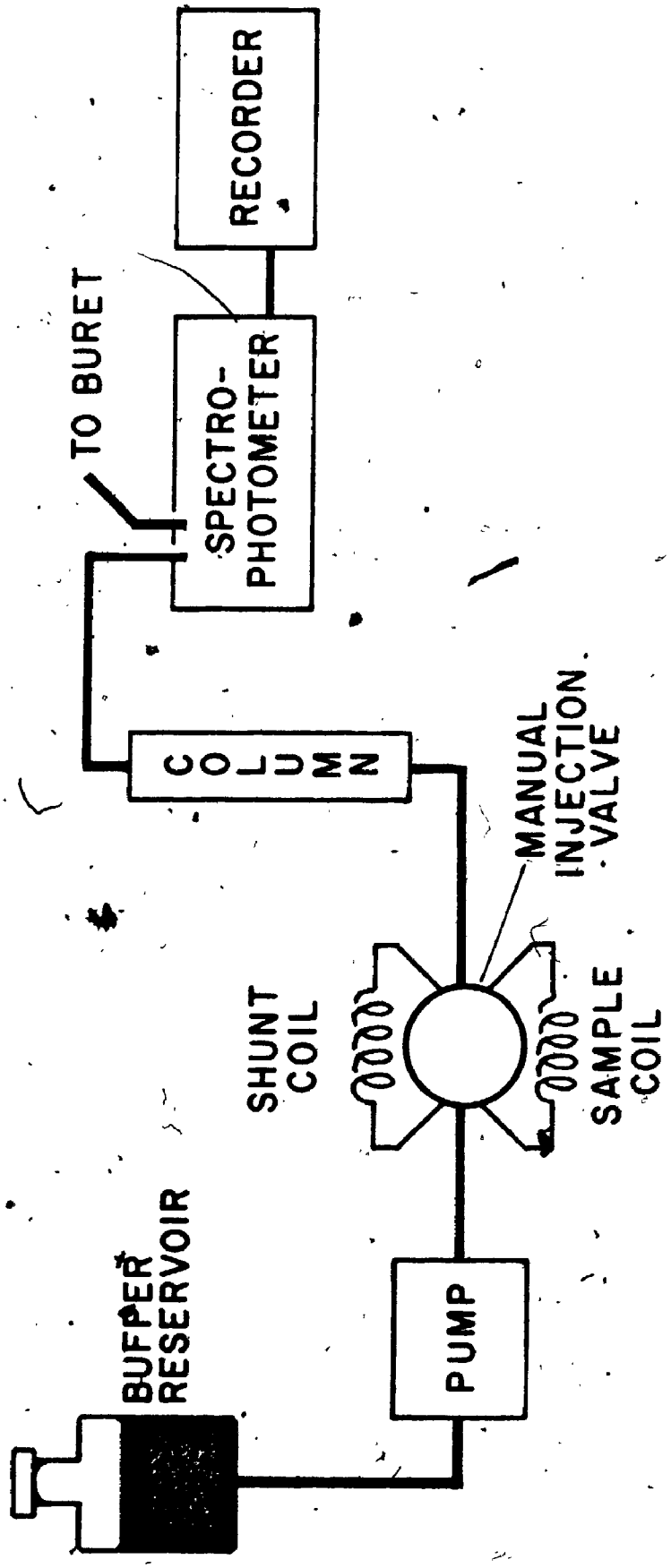
Sedimentation velocity at 59,780rpm and conventional sedimentation equilibrium runs at 20,410 rpm with 29,500 rpm overspeed were made with careful adherence to recommendations outlined in A Manual of Methods by C.H. Chervenka using various ionic strength conditions near 6 and 20°C. The instrument was a Beckman Model E analytical ultracentrifuge equipped with a Photoelectric Scanner calibrated at 368 nm. The buffer employed in all cases was PO_4-EDTA.

Gel Filtration Plateau Studies

The plateau study technique involves monitoring the elution profile created by the passage through a gel filtration column of a sample of sufficient volume to establish a region of constant concentration between the leading and trailing edges. To this end, an apparatus was set up as schematically presented in Figure 18.

Buffer from a four liter reservoir was pumped through either a 500 ul shunt coil or a 15 ml sample coil and into a 0.9 X 25 cm Chromatronix column packed with G-75 Fine Sephadex gel. The use of a Beckman Manual Injector Valve made possible the in-line selection of either coil and the filling of the other by syringe. Column effluent was passed through a Beckman DU Spectrophotometer fitted with a flow cell, collected in a buret and later discarded. The spectrophotometer was in turn electrically connected to a Sargent-Welch Model SRLG Recorder which plotted the elution

Figure 18: Schematic diagram of gel filtration plateau study apparatus.


RECORDER
SPECTRO-
PHOTOMETER
TO BURET
COLUMN
MANUAL
INJECTION
VALVE
SHUNT
COIL
SAMPLE
COIL
PUMP
BUFFER
RESERVOIR

profile.

An essential feature of the technique is the maintainance of constant flow rate. Therefore a Milton Roy Mini Pump or Beckman Accu-Flow Pump was used and the accumulating volume of effluent in the buret was recorded periodically as a function of time. Another salient feature is the injection of sample which is of constant composition. Thus sample mixing was minimized by the use of thin bore teflon tubing throughout and the intentional use of a short length between the sample coil and the column. The volume of sample applied was in all cases less than 12.5 ml of the 15 ml sample coil capacity, after which the injector valve was switched to the shunt coil prefilled with column buffer. This eliminated that part of the sample material in the sample coil that was in constant contact with and therefore diffusion mixed with the displacing buffer. Thus identical leading and trailing edges of the injected sample were assured. Flow was continued until the recorded absorbance had returned to baseline. Upward flow was employed. The pump position was chosen so as to pump into rather than out of the sample coil or column.

Samples intended to investigate the effect of ionic strength were diluted 2:1 with 3 M NaCl in PO_4-EDTA to achieve a sodium chloride concentration of two molar and then to a suitable protein concentration with 2 M NaCl in PO_4-EDTA.

Gel Filtration Supra Plateau Studies

An apparatus identical to that of the plateau studies was employed except that the 25 X 0.9 cm column was replaced with one of extended length (60 X 0.9 cm). A different sample coil and switching routines resulted in the sequential injection of three samples. The first was a hemoglobin solution. This was followed by a solution of hemoglobin and haptoglobin, with hemoglobin in excess. The third was a hemoglobin chase solution of concentration equal to that of the first.

The volume of the first sample was chosen to establish a plateau throughout the length of the column. That of the second was sufficient to allow migration of the complex it contained, being of greater Stokes radius with respect to hemoglobin, from this region into that of the first and to form a plateau superposed upon it. The volume of the third was less critical and served only to provide a trailing plateau of height equal to the first thus allowing investigation of the second sample's excess hemoglobin region left in the wake of the more rapidly migrating complex.

The sample coil was 1.6 mm inner diameter tubing of 39.5 ml capacity. This non-capillary bore was necessary so that flow resistance remained within acceptable limits. However, it was essential that the samples be of homogeneous composition. To this end, several air spaces served to isolate the sample in the coil from the displacing buffer. Also, the sample volume injected was invariably less than

85% of the solution volume within the coil. The shunt coil, prefilled with the hemoglobin solution, was pumped only briefly after switching it in-line to permit solution replacement in the sample coil.

The applicability of the experimental design depended upon the obtainable range of heme to haptoglobin molar ratio while the sensitivity was a function of the difference between the plateau and supra plateau heights, from which the concentration of haptoglobin bound monomer could be calculated. Thus sample concentrations were such as to approach but not exceed 0.8 optical density, this being the upper limit of the spectrophotometer range of linearity. The second sample was designed so that subsequent to the formation of complex the free hemoglobin remaining closely approximated that of the first sample. Four monomers were assumed to bind to haptoglobin. When α subunits were substituted for hemoglobin the degree of binding was assumed as two. The specifics of an experimental design involving an α:Hp molar ratio of 8.0 are presented in Table 2.

It was desirable for the hemoglobin or its α monomer to be near the centre of the separation range of the Sephadex gel employed to effect the chromatographic separation and yet have their haptoglobin complexes excluded from the gel matrix. This would ensure maximum mobility of the latter and avoid any partial resolution of metastable forms of $Hp\alpha_n$. Thus G-100 Fine was used in the case of hemoglobin, G-75 Fine in the case of α subunit.

Table 2: Details of a supra plateau study experimental design involving an α:Hp molar ratio of 8.0. The assumed number of α monomers bound by Hp was two.

Sample Number	[α] (uM)	[Hp] (uM)	Molar Ratio α/Hp	Optical Density	Absorbance α Bound
1	4.8	0.0	----	0.6	----
2	6.4	0.8	8	0.8	0.2
3	4.8	0.0	----	0.6	----

Determination of Number of α^{SH} Bound to Hp

A. Analytical Ultracentrifuge Technique

The photoelectric scanner of the model E yields calibrated graphs of optical density versus position in the cell from which the absorbance and hence concentration of components present can be derived. These scans were used to determine the number of α subunits bound by haptoglobin.

The zero-time scan of sedimentation velocity runs involving samples of various molar ratios of α:Hp established the total subunit concentration. In a like manner, the concentration of free α^{SH} was obtained from a later scan made when the haptoglobin-α subunit complex had sedimented sufficiently to leave a plateau of unbound α^{SH}.

Calculations of the molar ratio initially present in the sample and the number of α subunits bound per molecule of haptoglobin in these conditions were made as follows:

a. Molar Ratio $\alpha:Hp = \dfrac{[\alpha]_{total}}{[Hp]_{sample}}$

where $[\alpha]_{total} = (O.D._{total})/\varepsilon$

$[Hp]_{sample} = [Hp]_{stock} \times$ Dilution Factor

b. $\#\alpha / Hp = \dfrac{[\alpha]_{complexed}}{[Hp]_{sample}}$

where $[\alpha]_{complexed} = (O.D._{total} - O.D._{free})/\varepsilon$

A standard 1.2 cm path length sample cell was used. No correction for radial dilution was made.

B. Supra Plateau Study Technique

As described above, this technique involved the gel filtration of three sample regions of similar concentration of α subunit but with haptoglobin and therefore $Hp\alpha_n$ complex in the second only. This complex, being of greater mobility with respect to non-bound α^{SH}, moved ahead of the free α subunit thereby leaving a trough in its wake and creating a minor plateau superposed upon the plateau established by the first sample. From the difference in height of the supra plateau and plateau regions the concentration of bound α subunit could be calculated. The haptoglobin concentration was that present in the second sample and was therefore known. Hence an estimate of the number of α^{SH} bound per Hp could be made. The molar ratio α:Hp which defined the equilibrium conditions being studied was directly obtainable from the concentration of haptoglobin and of total α^{SH} indicated by the height of the supra plateau region above baseline.

Reaction of α^{SH} with Ethyl Acetimidate

Hemoglobin α^{SH} subunits were reacted with ethyl acetimidate hydrochloride using methods based on those of Wolfsy and Singer (1963) and Lockhart and Smith (in press).

Crystalline ethyl acetimidate was added in small lots to a solution of the subunits in PO_4-EDTA buffer pretitrated to pH 9.5 with 0.1 N NaOH. The reaction was performed at room temperature and with gentle stirring.

After each addition of reagent, the pH was adjusted to 9.5 with 0.1 N NaOH. Protein molarity was equal to or less than the 1.5×10^{-5} M corresponding to the 0.1 per cent concentration of hemoglobin recommended by Lockhart and Smith (in press). Sufficient ethyl acetimidate was used to obtain a ten fold molar ratio of reagent to target group, based on eleven lysyl residues per α subunit. After one hour, a considerable excess of crystalline glycine was added to terminate the process.

Reaction of α^{SH} with Dimethyl Adipimidate

The crosslinking of α subunits with dimethyl adipimidate dihydrochloride was performed according to the methods of Hartman and Wold (1967) and Lockhart and Smith (in press).

Protein concentration, molar ratio, and reaction conditions were identical to those described above with ethyl acetimidate except that 0.1 M sodium borate - 2×10^{-5} M EDTA pH 9.5 buffer was infrequently used in place of PO_4-EDTA.

Reaction of (Hp + α^{SH}) with Dimethyl Adipimidate

Procedures were again identical to those described above for reaction of ethyl acetimidate with α subunits. A protein solution was first prepared with an appropriate molar ratio of α^{SH} to Hp, and then diluted to a total protein molarity of 1×10^{-5} M or less. To calculate the amount of DMA required, the number of lysyl residues per

macromolecule was taken as 66 for Hp (Black et al, 1970) and 11 for α^{SH} (Dayhoff, 1969).

RESULTS AND DISCUSSION

PROTEIN PREPARATION

HAPTOGLOBIN PURIFICATION

Introduction

It was of considerable importance to obtain a haptoglobin preparation of near purity for two reasons. Extraneous protein contributing to the 280 nm absorbance would make numerical estimates of Hb subunits bound to Hp based on 412/280 absorbance ratios imprecise. Also, electrophoresis and TLG patterns would be further complicated with additional bands.

Haptoglobin, partially purified by the methods described earlier (denote as single G-200 purified Hp) was therefore ultrafiltered and chromatographed a second time resulting in the following elution profile (Figure 19). An ORTEC slab gel PAGE study showed that essentially the whole Hp region was contaminated with a protein of slightly higher molecular weight but much greater electrophoretic mobility. The identity assigned to this material was dimer albumin based on the following:

1. An ability, like that of monomer albumin, to absorb

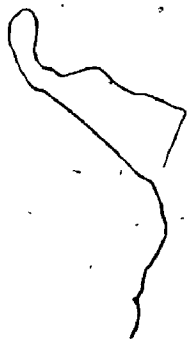


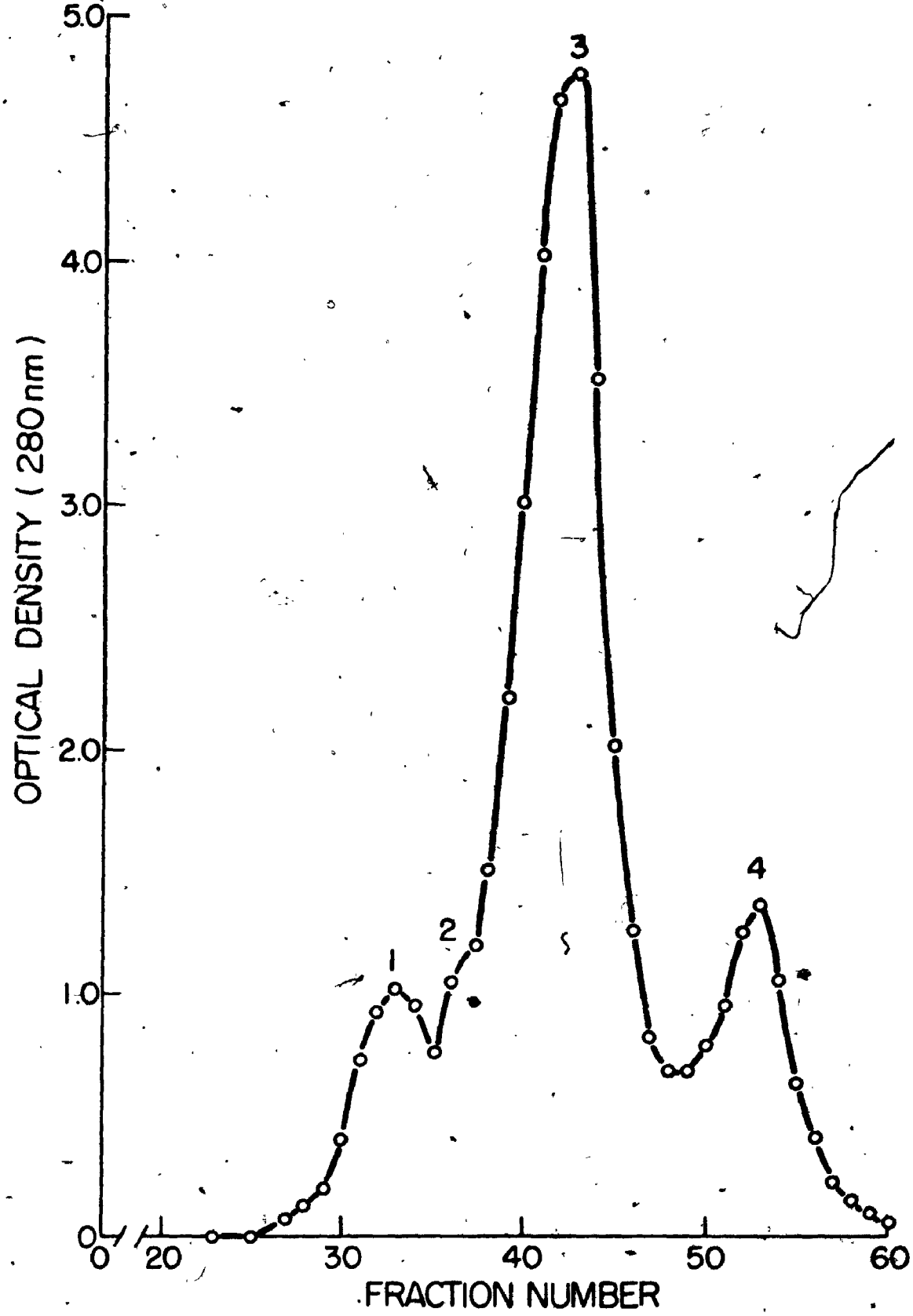
Figure 19: Elution profile resulting from second passage of partially-purified haptoglobin through a 5 X 100 cm Sephadex G-200 SF column.

The components were assigned the following identity: 1. Immunoglobulin

2. Evidence of dimer albumin

3. Haptoglobin

4. Monomer albumin


5.0
4.0
3.0
2.0
1.0
0
OPTICAL DENSITY (280 nm)
0
20
30
40
50
60
FRACTION NUMBER
1
2
3
4

and retain bromophenol blue tracking dye during electrophoresis.

2. An apparent molecular weight in Sephadex gel larger than that of Hp (100,000) and consistent with twice that of serum albumin (2 X 66,000 or 130,000).
3. Large net negative charge at pH 8.9 indicated by its rapid electrophoretic migration toward the anode.
4. Broad elution behavior in Sephadex implying intermolecular association with itself and monomer albumin.
5. An increased presence with time and concommitant decrease in monomer albumin in stored partially-purified Hp samples.

All of the above are consistent with the properties of dimer albumin as reviewed by Foster (1960).

Although freshly drawn pig blood contained minimal amounts of dimer albumin it was not practical to obtain small volumes frequently and immediately carry the purification of the Hp they contained to completion. In addition, gel filtration seemed inadequate to remove the dimer albumin formed during storage of a larger amount of semi-pure Hp. Acidification to pH 4.7 was a necessary step in the DEAE-cellulose purification and was known to increase the association of monomer to dimer (Foster, 1960). The addition of non-denaturants which might decrease this association was considered. For example, long chain fatty acids might become

imbedded into hydrophobic regions of the albumin subsurface with protruding charged ends introducing repelling charges into the association interface, but it was realized that once the disulfide bridge linking the single sulfhydryl of each albumin monomer was spontaneously formed, nothing short of reductive cleavage with β-mercaptoethanol or dithiothreitol would be effective in elimination of this troublesome contaminant. This unfortunately would also destroy Hp functionality due to the susceptibility of its disulfide linkages to reduction (Lockhart, Chung and Smith, 1972).

Introduction of a G-100 Sephadex step (9.5 X 50 cm column) after DEAE-treatment and prior to G-200 chromatography which was designed to remove monomer albumin at an earlier stage to limit the formation of dimer albumin was judged to be unsuccessful since considerable dimer was already present and the fraction pooling necessary to eliminate it would result in unacceptable losses of Hp. Figure 20 shows the profile of G-100-purified Hp eluted from a 5 X 100 cm G-200 SF column. Figure 21 illustrates the banding pattern seen in an ORTEC gel slab of samples taken from fractions across the profile.

Further methods were considered to take advantage of the dissimilarities of haptoglobin and dimer albumin.

Prep-Disc Electrophoresis of Haptoglobin

Prep-Disc electrophoresis of 20 mg of a protein preparation that was approximately 80% haptoglobin and

Figure 20: Elution profile of Sephadex G-100 partially purified Hp through a 5 X 100 cm Sephadex G-200 SF column.

The components were assigned the following identity: 1. Immunoglobulin

2. Haptoglobin

3. Monomer albumin

The leading edge assymetry of the second peak was considered due to the presence of dimer albumin.

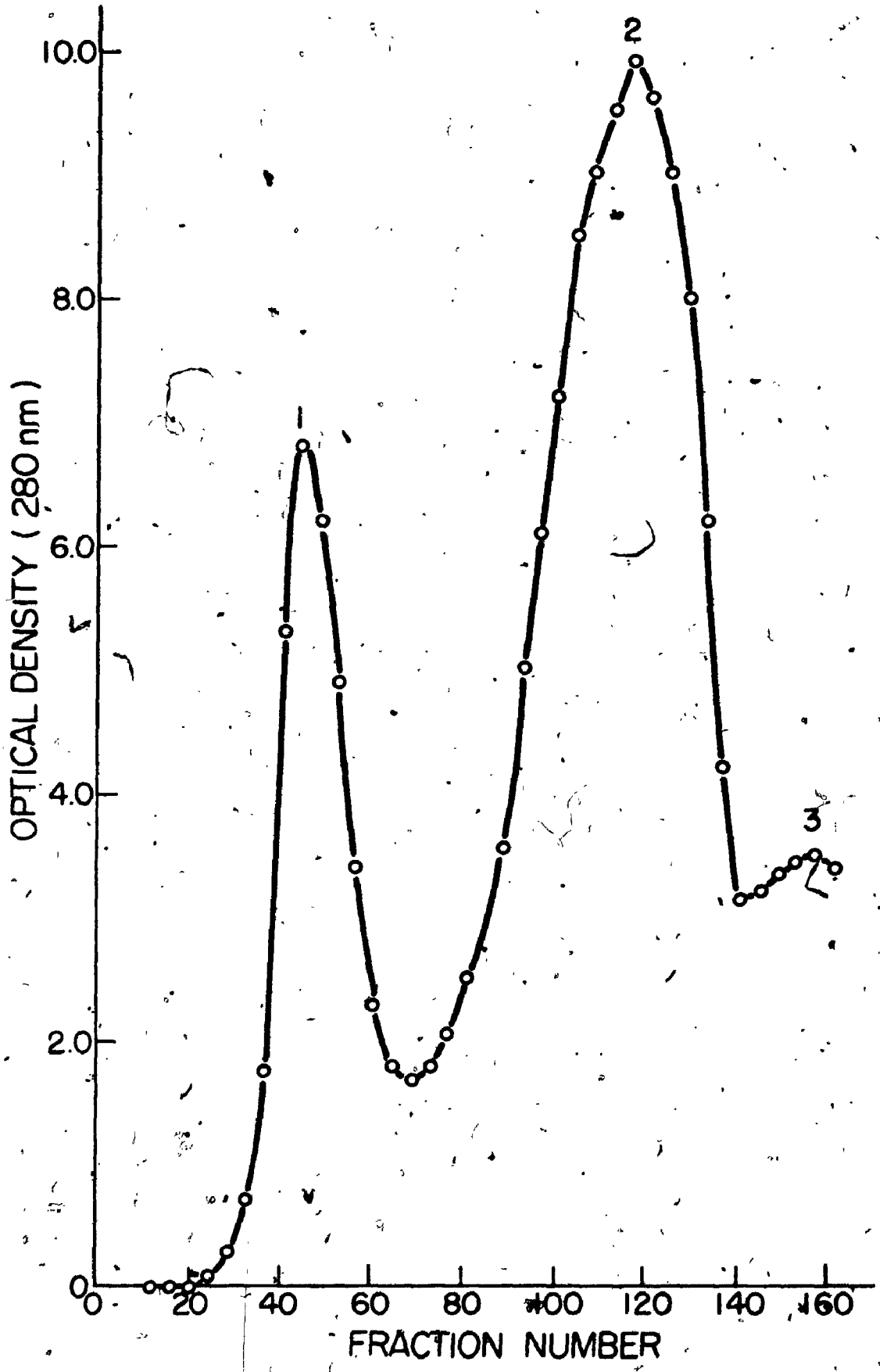

10.0
8.0
6.0
4.0
2.0
0
OPTICAL DENSITY (280 nm)
2
3
0
20
40
60
80
100
120
140
160
FRACTION NUMBER

Figure 21: Slab gel electrophoresis pattern of eluant fractions derived from Sephadex G-200 SF column chromatography of Sephadex G-100 partially purified haptoglobin (Figure 20). Fraction number increases from left to right. Proposed band identities are as follows:

1. Immunoglobulin
2. Haptoglobin
3. Dimer albumin
4. Monomer albumin

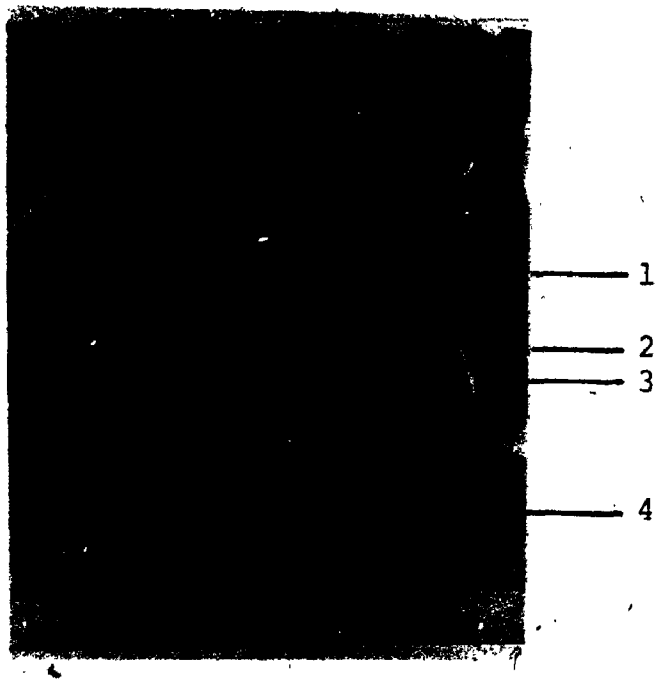
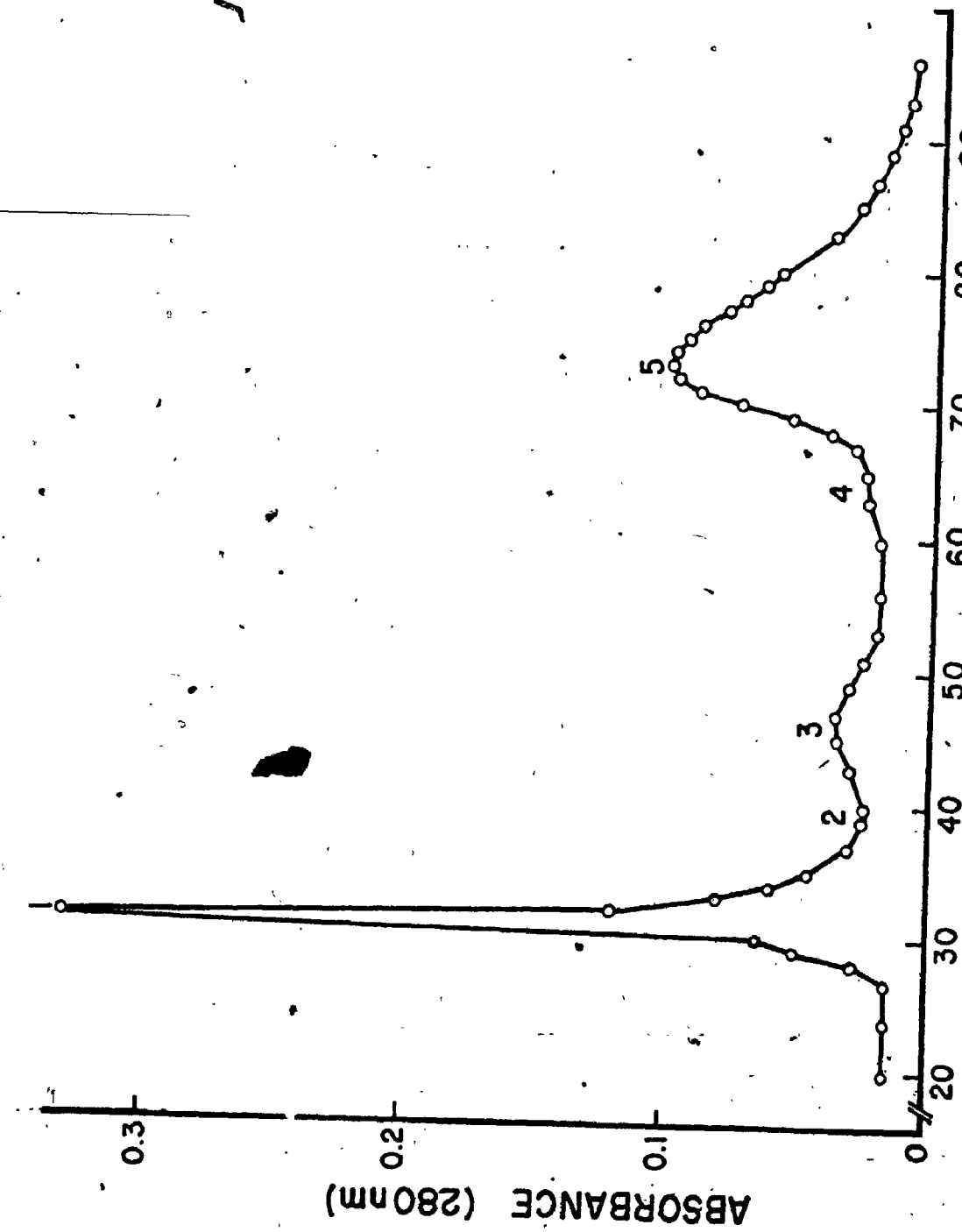

1
2
3
4

Figure 22: Prep-disc electrophoresis profile of 15 mg of 80% pure haptoglobin.

Component identities assigned were as follows:

1. Acrylamide not incorporated into gel matrix
2. Bromophenol blue (optically visible)
3. Monomer albumin
4. Dimer albumin
5. Haptoglobin


ABSORBANCE (280nm)
0.3
0.2
0.1
0
20
30
40
50
60
70
80
90
FRACTION NUMBER
2
3
4
5

10% dimer albumin resulted in the elution profile of Figure 22. A larger sample size or dimer albumin content resulted in overlap of the haptoglobin and dimer albumin regions. These sample limitations coupled with the relative sophistication involved and care required to successfully apply this technique prompted the investigation of affinity chromatography as an alternative method of preparative scale haptoglobin purification.

Affinity Chromatography of Haptoglobin

Single G-200 purified Hp fractions intended for affinity chromatography with Concanavalin A-Sepharose 4B were pooled disregarding albumin content but with careful exclusion of immunoglobulin material to avoid the presence of any non-Hp glycoprotein. Due to the nature of the semi-pure Hp G-200 profile (Figure 17) very little Hp was lost. This preparation was designated Hp-Alb.

The following experiments were all conducted at 5°C.

A preliminary study was undertaken to investigate the carbohydrate monomer concentration required to release Hp bound to the Con A matrix. One ml of Con A-Sepharose 4B was exposed to an excess of Hp as Hp-Alb and twice to five ml of increasing concentrations of α-methyl-D-mannoside. After each five ml addition, it was briefly vortex-mixed three times during a 5 minute period, and then centrifuged at 7000 rpm for 10 minutes. The supernatant was drawn off without gel inclusion and its absorbance at 280 nm deter-

mined (Table 3). Initial washing with 0.1 M NaCl removed non-bound protein. Contrary to the expected trend, there was a gradual removal of protein as indicated by the second treatment at a given mannoside concentration resulting in an equal or increased supernatant protein content with respect to the first. Also there was no critical concentration for a dramatic protein release.

A 1 cm diameter test column containing a 10 ml bed volume of Hp-saturated Con A-Sepharose 4B was prepared and washed with 10 ml of 0.1 M NaCl, 20 ml of 0.1 M NaCl in 0.02 M phosphate pH 7.2 (PO_4-NaCl) and eluted with 350 ml of 0.1 M α-D-glucose (GLU) followed by 150 ml of 1.0 M GLU in PO_4-NaCl. Fraction volumes collected were approximately 4 ml and their absorbance was read at 280 nm (Figure 23). Since the elution profile was found to be very broad, fractions were pooled in 50 and 20 ml quantities and concentrated to 5 ml by ultrafiltration. To minimize solute concentration differences which affected elution profile baseline, twenty ml of distilled water was then added and ultrafiltration was continued to a volume of 3 ml. Figure 24 graphically presents protein release.

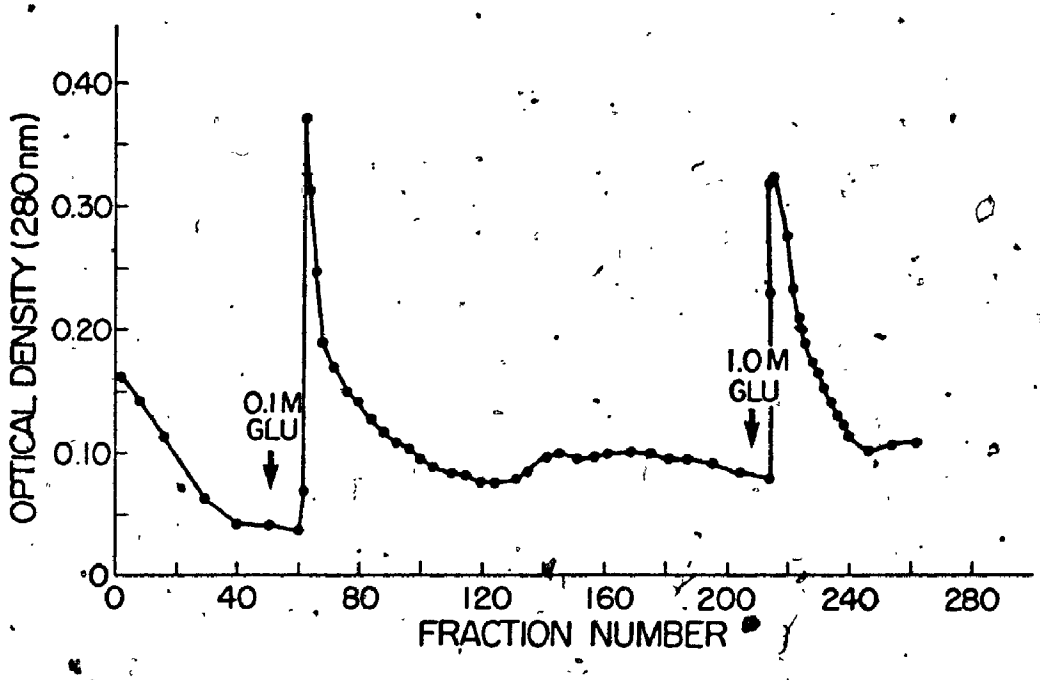
To estimate Hp binding efficiency and capacity, 100 ml of fresh Con A-Sepharose 4B was packed in a 2.5 X 25 cm column and two 50 ml samples of semi-pure Hp, separated by 200 ml of buffer wash, were applied. Hp content of fractions collected, as determined by Hb binding capacity, was found to be much lower than that of the samples applied but

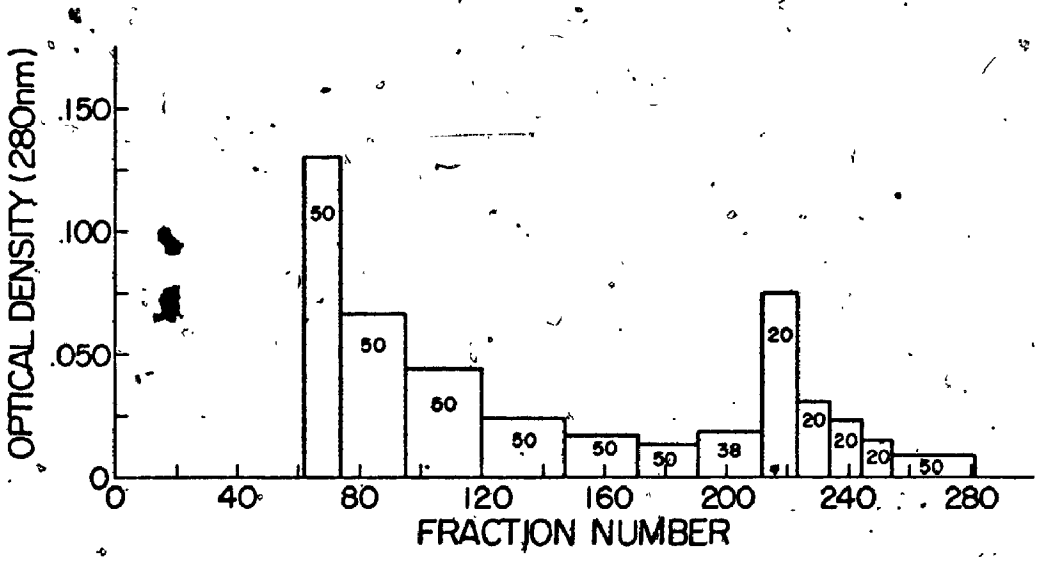
Table 3: Stepwise removal of haptoglobin bound to 1 ml of Con A-Sepharose 4B gel.

Solution Added	Concentration	Resulting Absorbance (280 mu)
NaCl	0.01 M	0.067
	0.01 M	0.018
Mannoside	0.05 M	0.100
	0.05 M	0.122
	0.10 M	0.085
	0.10 M	0.070
	0.25 M	0.048
	0.25 M	0.080
	0.50 M	0.050
	0.50 M	0.064
	0.75 M	0.050
	0.75 M	0.069

Figure 23: Stepwise elution of Hp off Con A-Sepharose 4B gel. Starting positions of 0.1 M and 1.0 M glucose are indicated by arrows.

Figure 24: Absorbance of pooled fractions of protein stepwise-eluted off Con A-Sepharose 4B after ultrafiltration to a common volume (3 ml). The volume of the pools before ultrafiltration are given in the rectangles.


OPTICAL DENSITY (280nm)
0.40
0.30
0.20
0.10
0
0.1 M GLU
1.0 M GLU
0
40
80
120
160
200
240
280
FRACTION NUMBER


OPTICAL DENSITY (280nm)
.150
.100
.050
0
50
50
60
50
50
50
38
20
20
20
20
50
0
40
80
120
160
200
240
280
FRACTION NUMBER

increased across the fractions derived from the second sample and was also significant in those of the first. Linear gradient elution with 0.0 to 0.1 M glucose, total volume of 500 ml, gave a very broad profile (Figure 25). These findings created serious doubts as to the advisability of using common column techniques.

A batch method was therefore investigated. Con A-Sepharose 4B was incubated with Hp-Alb for six hours with gentle stirring and for twenty-four hours without, and then packed in a column. After washing out unbound protein, the gel was then suspended in 0.5 M glucose with gentle stirring for six hours, repacked as a column and the released protein washed out. Repetition of the glucose incubation released no further protein based on the 280 nm absorbance of the supernatant. PAGE of an aliquot of the Hp-Alb solution before and after first exposure to Con A shows the effectiveness of adsorption (Figure 26) while purity of the Hp eluted off the Con A-Sepharose 4B is demonstrated in Figure 27.

Discussion of Haptoglobin Affinity Chromatography

Several conclusions of interest were made as the study developed. Preliminary investigations suggested that release of Hp from Con A binding sites would be gradual, and that no critical concentration existed which would result in wholesale replacement by carbohydrate monomer. A stepwise 0.1 to 1.0 M glucose elution showed that an initial burst of Hp,

Figure 25: Linear gradient elution of haptoglobin off Con A-Sepharose 4B. The gradient consisted of 0.0 to 0.1 M α-D-glucose, total volume of 500 ml.

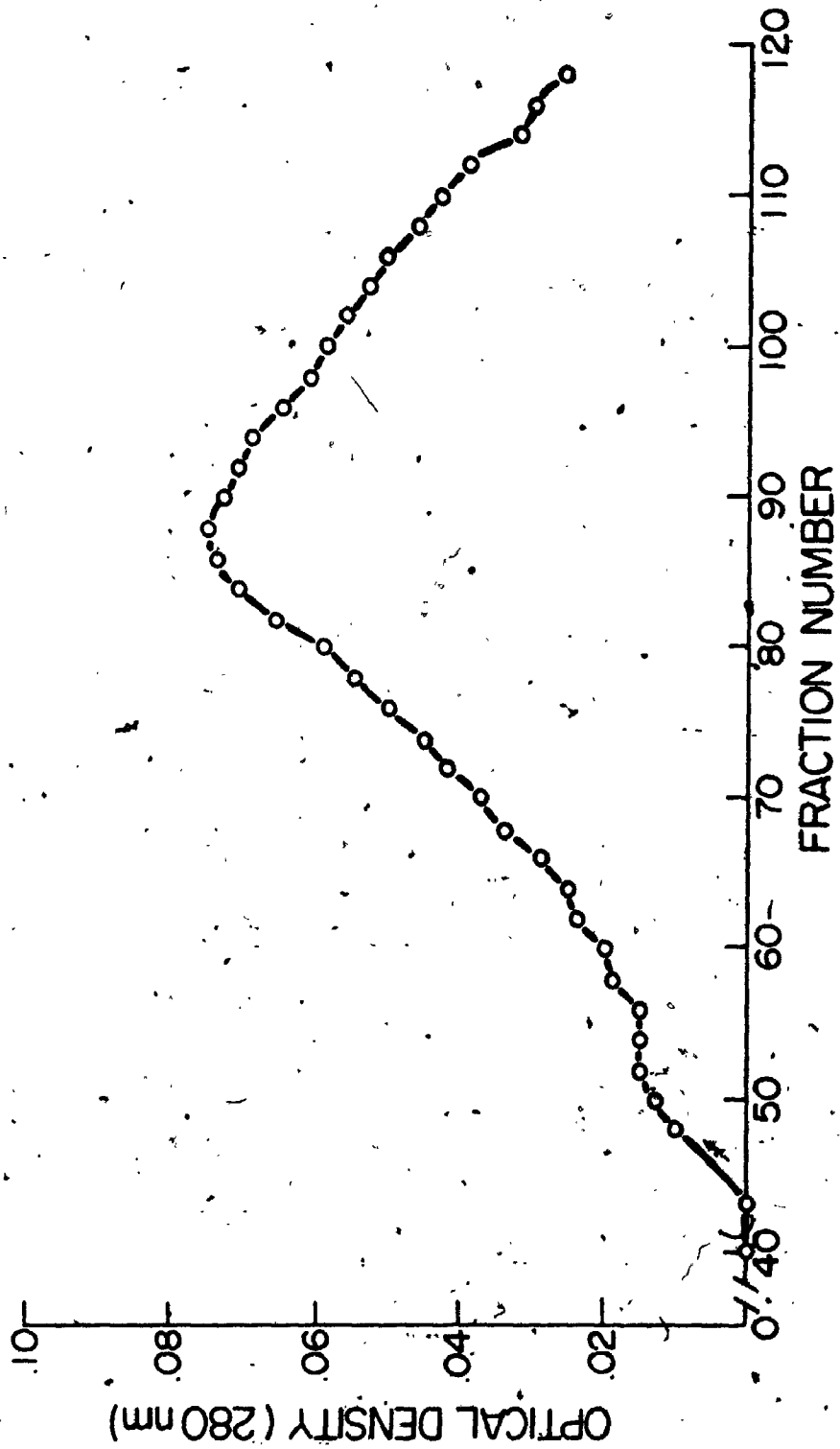

OPTICAL DENSITY (280 nm)
0.2
0.4
0.6
0.8
1.0
FRACTION NUMBER
40
50
60
70
80
90
100
110
120

Figure 26: Polyacrylamide gel analysis of the effectiveness of haptoglobin adsorption by Con A-Sepharose 4B. Assigned band identities are indicated. That of monomer albumin is at the threshold of visual detection.

Left: Semi-pure Hp solution before exposure to Con A-Sepharose 4B.

Right: The same solution after exposure to Con A-Sepharose 4B.

Figure 27: Polyacrylamide gel analysis of Hp purity after elution off Con A-Sepharose 4B.

Assigned band identities are indicated: That of immunoglobulin is at the threshold of visual detection.

HAPTOGLOBIN

DIMER ALBUMIN

MONOMER ALBUMIN

HAPTOGLOBIN

IMMUNOGLOBULIN

perhaps held to the gel by a single oligosaccharide chain, could be obtained but that the removal of further material was an ongoing process. Stepping the glucose concentration up to the higher level produced another burst of Hp followed by a second but diminished gradual release as elution neared completion.

When two samples were separately introduced into a column of Con A-Sepharose 4B, it was found that although maximal binding had not been reached by application of the first sample as evidenced by the further uptake of Hp from the second, some Hp was not retained. This fact coupled with the detection of a continually present and increasing amount of Hp in the eluant derived from the second sample implied that a saturation process was continuing and that it was approaching but still a significant distance away from completion. If this were not the case, no Hp would have appeared in the eluant until part way through the second sample and then the concentration would have increased rapidly to that of the sample before application.

These findings can be explained by considering the binding of individual Hp molecules to the gel as involving a number of points of attachment ranging from one to the theoretical maximum equal to the number of oligosaccaride chains present in pig haptoglobin (exact number is unknown but a reasonable estimate is seven). The particular number of attachments of each molecule would depend on factors such as proximity and three dimensional relationship of

Con A sites in the gel matrix, number of molecules vying for the sites in a common vicinity, and steric factors involving length and position of saccharide moieties. Exchange of oligosaccharide by monosaccharide is a time-dependent spatial process and it is conceivable that this exchange may be more difficult for some Hp binding orientations than others. In addition, if the Hp molecule is bonded to the matrix by several attachments, only one of which is necessary for its retention, the time available for monomer exchange by oligosaccharide drastically increases. All of these factors are thought to cumulatively explain the novel necessity of a batch purification approach with long incubation times. This facilitates maximum protein binding strength and capacity by providing ample opportunity for repeated adsorption, dissociation and resorption.

Turning our attention to protein elution, in view of the oligo- and monosaccharide competition for any binding sites which become available, a thousand fold mono- to oligosaccharide ratio coupled with the greater relative mobility of the monomer should, with sufficient time, result in virtual complete protein displacement. It seems appropriate to stress the time element when one considers the combined effect of the moderate binding strength involved (K_{EQM} for α-methyl-D-mannoside = 2 X 10^4 M^{-1} according to So and Goldstein, 1968) and the number of binding positions at which each protein is attached to the

gel matrix. Column elution profiles can be predicted to vary with flow rate as well as with monosaccharide concentration and temperature in systems where multi-position attachments are involved. It can also be predicted that such systems cannot give good resolution of different glycoproteins thereby necessitating, as in the present case, their removal by other means.

The capacity of the commercial Con A-Sepharose 4B for Hp was not experimentally determined due to sub-maximal amounts available for binding. The monosaccharide capacity can be estimated as 4.5×10^{-2} mg α-D-glucose/ml based on a molecular weight of 32,000 per Con A binding site (Yariv et al, 1968) and the stated Con A concentration of 8 mg/ml of swollen gel. However with due consideration of the above discussion, it is apparent that the haptoglobin binding capacity cannot be defined.

The haptoglobin derived from these isolation efforts was of near purity. The slight immunoglobulin contaminant could have been avoided by more propitious pooling of G-200 fractions to obtain the Hp-Alb material. Therefore 100% purity, on a large scale, is clearly practical.

Microheterogeneity of Haptoglobin

Many of the microheterogeneity phenomena of human haptoglobin 1S-1S, due at least in part to sialic acid content, reported by Yang and Przbylska (1973) were observed in this study with porcine haptoglobin. They found eight

bands in freshly prepared Hp but that this number increased upon storage at 4°C. Porcine Hp stored in excess of one month consisted of at least fourteen components (Figure 28) all of which were capable of binding Hb and reduced to a narrower less defined group upon treatment with neuraminidase. Compared with standard methods of PAGE, enhanced separation of Hp bands was achieved by combining the TRIS-glycine pH 8.9 electrode buffer of Ornstein and Davis (1964) with the TRIS-diethylbarbituric acid pH 7.8 gel system based on that of Williams and Reisfield (1964). Monomer albumin was seen as a narrow band at or close behind the ion front while dimer albumin was more diffuse but also highly mobile. Both were well separated from the Hp components detected. Investigation by this hybrid PAGE method of the leading and trailing edges of the Hp region resulting from G-200 Sephadex chromatography of a semi-pure preparation indicated some resolution (Figure 29) with gel filtration mobility associated with lower electrophoretic mobility. This is consistent with a concept of carbohydrate moiety loss with time.

Affinity chromatography does not seem as suitable for separation of Hp components as PREP-DISC electrophoresis. Although gradient elution of Hp bound to Con A-Sepharose 4B might yield a partial fractionation according to carbohydrate content, this was beyond the scope of this work and therefore not pursued.

Figure 28: Polyacrylamide gel analysis of haptoglobin microheterogeneity using a hybrid system.

Figure 29: Haptoglobin microheterogeneity as a function of gel filtration mobility through a 5 X 100 cm G-200 SF Sephadex column. Investigation was by polyacrylamide gel electrophoresis of the Hp region of the elution profile.
Left: Leading edge.
Right: Trailing edge.
Band identities assigned are as indicated.

HAPTOGLOBIN

HAPTOGLOBIN

DIMER ALBUMIN

MONOMER ALBUMIN
(narrow band)

Preparation of α^{SH} and α^{PMB} Subunits

In their paper describing the preparation of hemoglobin subunits, Geraci et al (1969) alluded to instability in the 10 mM sodium phosphate pH 6.6 conditions of the CM-cellulose step and found early elution with 20 mM TRIS pH 8.0 advisable. Conditions of 0.1 M sodium phosphate pH 7.5 seemed better suited for subunit stability as well as being capable of bringing about subunit elution.

Salvati et al (1969) have reported enhanced autoxidation of hemoglobin by metal ions, particularly copper. This was of particular concern as met α subunits tend to undergo extensive conformational change to form a hemochromagen which is much less stable than the native form (Bucci and Fronticelli, 1971). Therefore all solutions in their preparation and use were 2×10^{-5} molar in EDTA. This inclusion of a chelating agent, a departure from the method of Geraci et al (1969), follows the lead of Antonini, Bucci et al (1965).

The DEAE step was reported to give complete separation of α subunits from unreacted or partially modified hemoglobin, and seemed at first sight to do so in the hands of this author (Figure 30). However it was discovered that the protein contained in the first DEAE peak was not of a single molecular weight. Sephadex-G 75 chromatography was necessary to separate α^{SH} and α^{PMB} monomer from a higher molecular weight contaminant. Figures 31 and 32 show the completeness of separation routinely

Figure 30: Elution profile of DEAE-Sephadex column as part of the α subunit preparation.

The numbering of fractions was begun with the appearance of protein. Their volume was 5 to 6 ml.

Assigned identity of components:

1. α^{PMB} subunit
2. Partially and/or unreacted hemoglobin

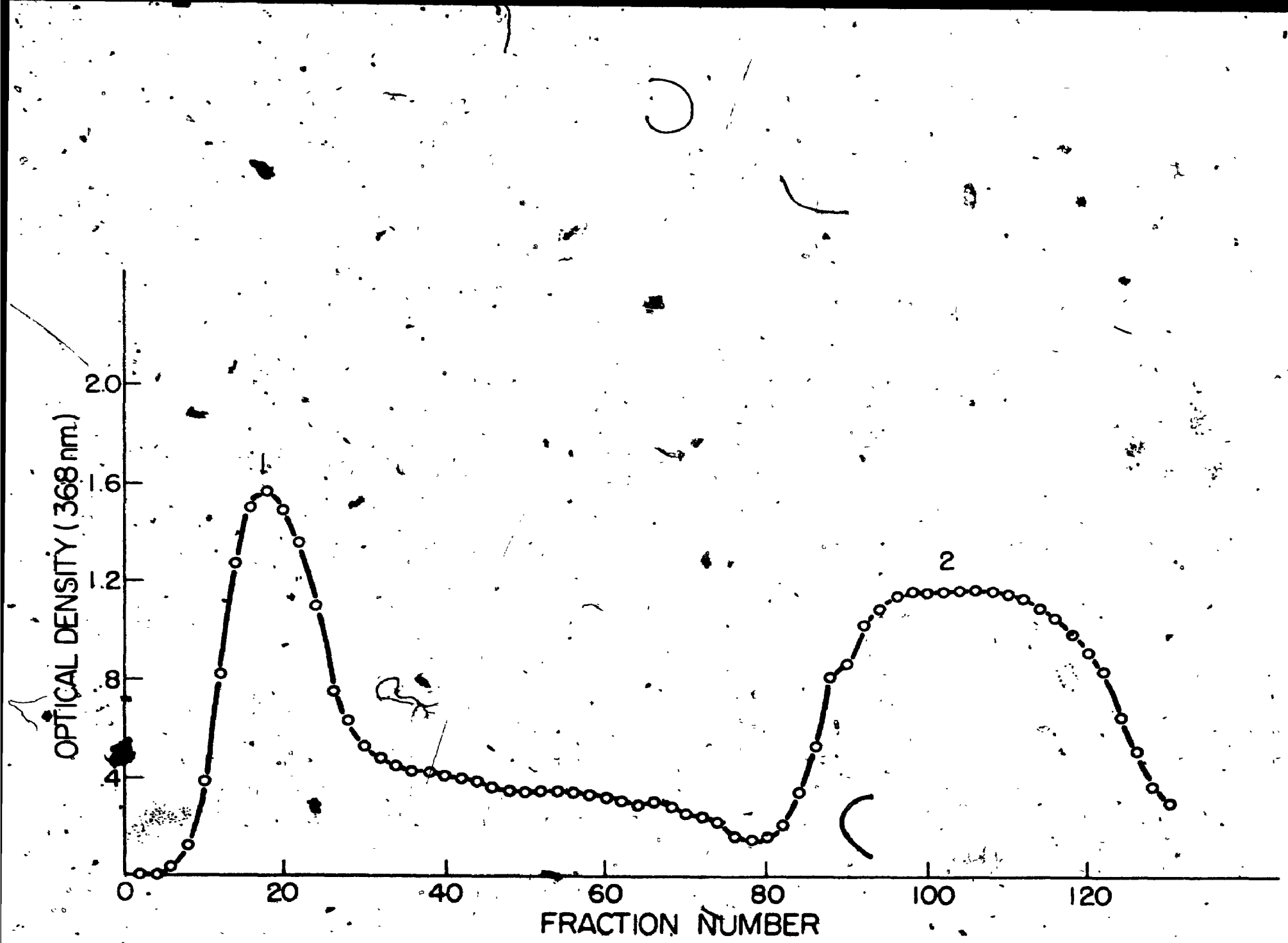
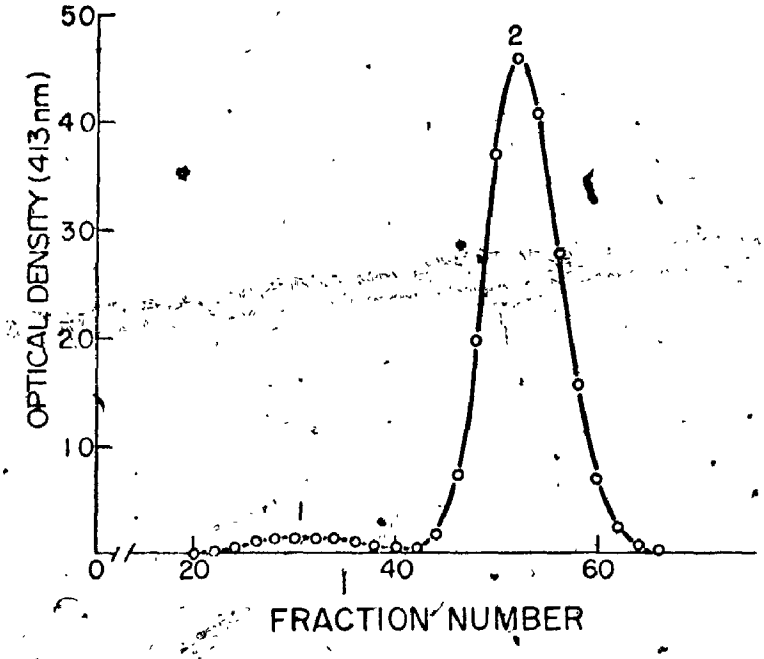

OPTICAL DENSITY (368 nm)
2.0
1.6
1.2
.8
.4
0
20
40
60
80
100
120
FRACTION NUMBER
1
2

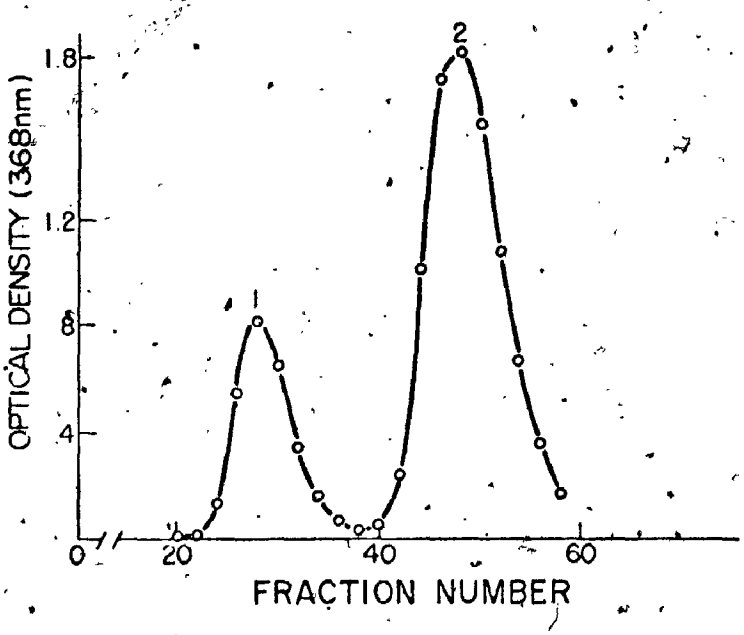
Figure 31: Elution profile of α^{PMB} chromatographed through a 2.5 X 90 cm G-75 Sephadex column equilibrated in PO_4-EDTA. The optical density of the fractions was determined at 413 nm and 368 nm where appropriate. The latter were converted to values at 413 nm using the respective extinction coefficients.

Assigned identity of components: 1. Contaminant
2. α^{PMB}

Figure 32: Elution profile of α^{SH} chromatographed through a 2.5 X 90 cm G-75 Sephadex column equilibrated in PO_4-EDTA. Peak positions do not coincide with those in Figure 31 due to a difference in fraction size.

Assigned identity of components: 1. Contaminant
2. α^{SH}


OPTICAL DENSITY (413nm)
50
40
30
20
10
0
20
40
60
1
2
FRACTION NUMBER


OPTICAL DENSITY (368nm)
1.8
1.2
.8
.4
0
20
40
60
1
2
FRACTION NUMBER

accomplished. The contaminant was present, albeit in lesser quantities, very early in the DEAE-elution since only the leading edge of this first peak was used as a source of α^{PMB}.

Identity of Subunit Contaminant

The nature of the contaminant was of sufficient importance to warrant investigation.

Its visible spectrum was identical to that of purified α-subunits. By integrating the area of the contaminant and α peaks resulting from the same sample being G-75 chromatographed 100 hours apart, the relative proportion of contaminant and monomer was found to be constant. Thus there was no net conversion.

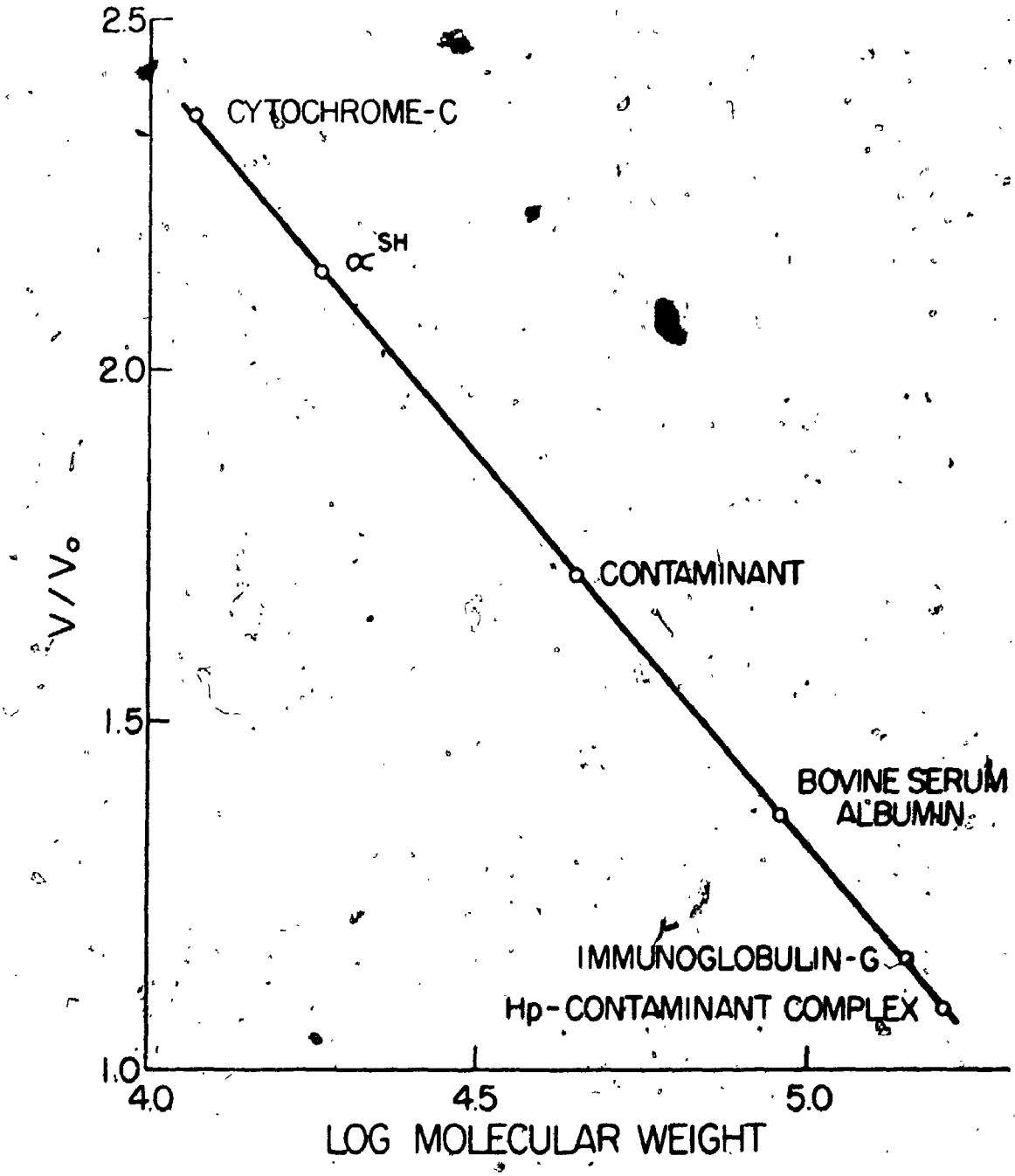
When the elution position of the contaminant was compared with that of stock HbO_2 in the Hp binding capacity system, they were found to coincide as did the complexes formed by each upon addition of Hp.

The contaminant was found to have an ability to bind to Hp equivalent to that of stock HbO_2 since when solutions of each were diluted to a common optical density at 413 mu and an equal amount of Hp added to each the resulting samples, studied by PAGE followed by densitometry, contained equal amounts of complex.

Cytochrome-C, bovine serum albumin, bovine immunoglobulin-G and hemoglobin α^{SH} subunits were used to calibrate a 2.5 x 45 cm G-150 superfine column. Figure 33

Figure 33: Molecular weight determination of the α^{SH} contaminant and its haptoglobin complex by gel filtration.

The calibration curve of a 2.5 X 45 cm G-150 SF Sephadex column was constructed using cytochrome C, α^{SH}, bovine serum albumin and immunoglobulin G as markers. The apparent molecular weight of the α^{SH} contaminant and its Hp complex was calculated as 44,700 and 162,500 respectively.


2.5
2.0
1.5
1.0
V/Vo
CYTOCHROME-C
α SH
CONTAMINANT
BOVINE SERUM ALBUMIN
IMMUNOGLOBULIN-G
Hp-CONTAMINANT COMPLEX
4.0
4.5
5.0
LOG MOLECULAR WEIGHT

shows the calibration curve with points corrected by the method of Whitacher (1963) and Leach and O'Shea (1965) for the chromatographic temperature being 5° rather than 20°C. The apparent molecular weights of the contaminant and its haptoglobin complex are 44,700 and 162,500 respectively.

Titration with PCMB yielded sulfhydrl per monomer values of 0.548 for hemoglobin and 0.595 for the contaminant, or 2.19 and 2.39 -SH per tetramer. These values compare favourably with the hemoglobin literature value of 2.39 (Benesch and Benesch, 1962).

The amino acid composition of gel filtration-purified contaminant was determined. Due to its low concentration in the eluant derived from the final purification step of α^{SH} subunits, sufficient material for only one long column analysis was obtained. Acid hydrolysis of large volumes was suspected of causing some decompositional error due to incomplete de-oxygenation. However comparison with stock hemoglobin analyzed under the same conditions indicated near identity. Agreement of the experimental and known compositions of hemoglobin was very good but involved some slight deviation as expected. See Table 4 for these findings. Analysis of column buffer indicated no ninhydrin positive material.

On the basis of the above, it can be concluded that a very minor component was invariably present in

3 5

OF/DE

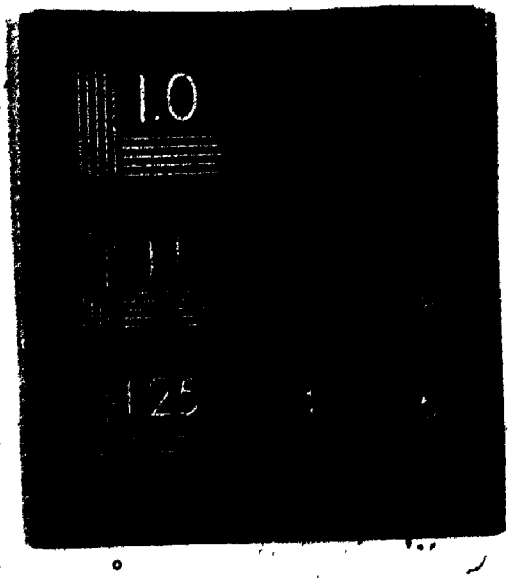


Table 4 : Amino acid analysis of α^{SH} subunit and its contaminant. Only long column analysis was performed on the latter due to a shortage of material..

Amino Acid	Known Composition			Observed Composition		
	α	β	Hb/2	α	C*	Hb/2
Ala	21	15	36	22	34	31
Arg	3	3	6	3		
Asp,Asn	12	13	25	12	30	28
Glu,Gln	5	11	16	5	18	18
Gly	7	13	20	7	21	21
His	10	9	19	9		
Ile	0	0	0	0	0	0
Leu	18	18	36	18	38	38
Lys	11	11	22	11		
Phe	7	8	15	7	14	14
Pro	7	7	14	7	14	14
Tyr	3	3	6	3	4	5
Val	13	18	31	13	30	27

C* Contaminant

α^{SH} subunits prepared according to the method of Geraci, Parkhurst, and Gibson (1969). Physical properties, ability to bind haptoglobin, and amino acid composition indicate that this material is in fact hemoglobin. Its presence in subunit preparations, or the possibility thereof, necessitate sensitive analysis of molecular weight homogeneity and, as found during the course of this work, the inclusion of a gel filtration purification step.

This begs the question as to whether some of the previous work in this field was marred by undetected impurity. Nagel and Gibson (1967) reported limited binding of their α^{PMB} preparations to haptoglobin as seen by electrophoresis. No blank run was made. Chiancone, Alfsen et al (1968) did include a blank in which the α^{PMB} was apparently pure. However it should be pointed out that a very minor contamination, that is, of the order of less than five per cent of the protein applied, would not be detected. Several studies involve sedimentation in the ultracentrifuge. Here again, such a slight amount of concentration might not be seen. A number of different methods of α subunit preparation have been employed but in all cases proof of purity has been inadequate.

It is significant that the contaminating material was not found to be a non-reversibly and continuously forming aggregation product of α^{SH}. That would predicate

much study of its nature, prevention of its formation, and proof of removal. Before this was possible, no meaningful investigation of the interaction of haptoglobin and the native monomer of hemoglobin could be undertaken.

Hemoglobin α Subunit Quality

By all methods of analysis employed, the G-75 Sephadex purified α^{SH} subunits were monomers with native conformation.

They possessed the 300 to 600 nm spectrum of oxy hemoglobin (Figure 34) with no sign of conversion to the met form during the normal time of use. Subunits rechromatographed through a G-75 Sephadex column one week after isolation showed no material of molecular weight larger than monomer, indicating the absence of irreversible aggregation or denaturation. Sulfhydryl titration yielded a value of 1.06 -SH per monomer thus proving the effectiveness of cysteine-104 regeneration by β-mercaptoethanol. There was excellent agreement of amino acid composition with the known (Table 4) and the α^{SH} subunits and in turn the ability to form saturated complex with haptoglobin. However β subunits of satisfactory quality were not easily prepared and this approach was thought to be beyond the scope of this investigation.

In contrast to α^{SH}, the α^{PMB} subunits were not electrophoretically pure (Figure 35). They consisted of one major component with mobility greater than that of

Figure 34: Absorption spectrum of α^{SH} subunit from 300 nm to 600 nm.

Experimental measurements were most often made at 368 nm and 413 nm. These wavelengths are indicated by arrows.

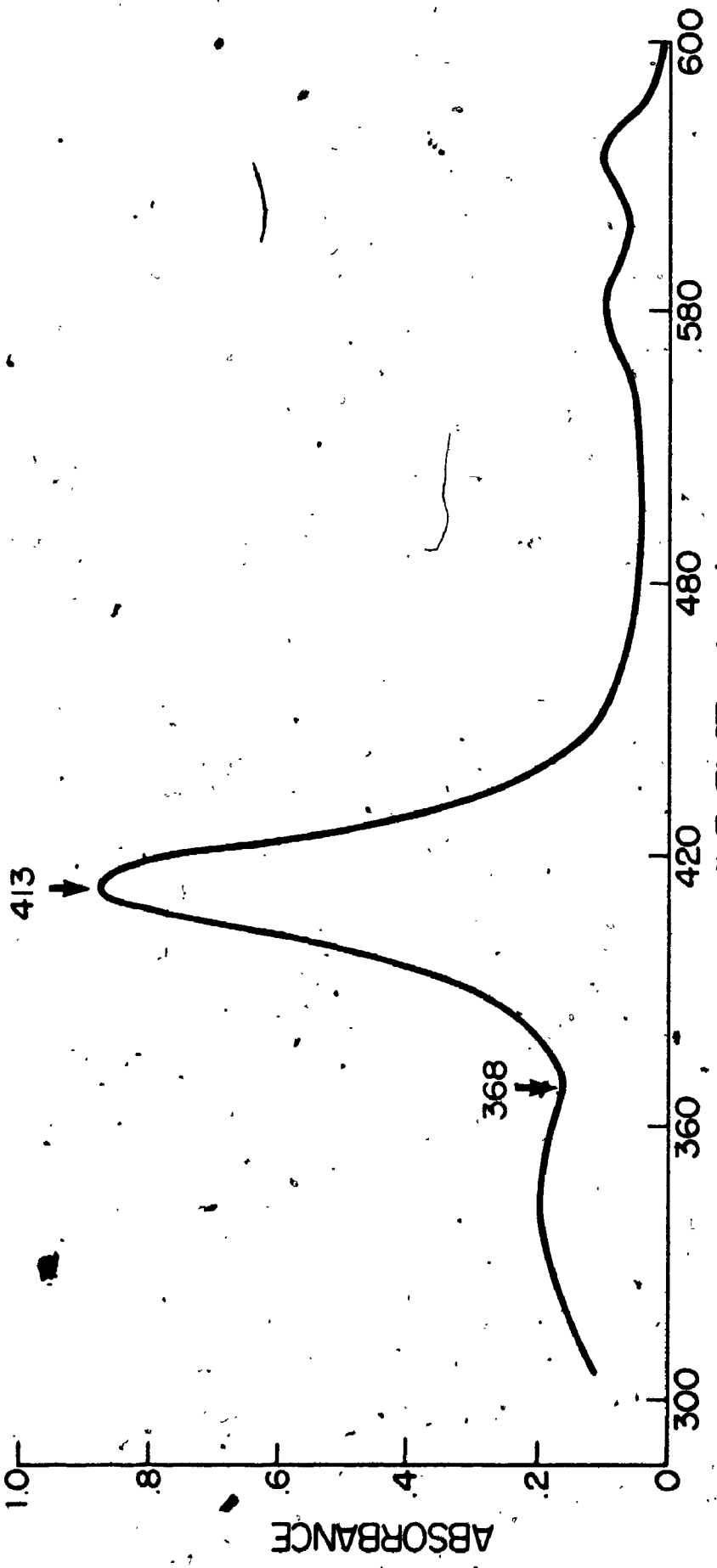

ABSORBANCE
1.0
.8
.6
.4
.2
0
300
360
420
480
580
600
WAVELENGTH (nm)
413
368

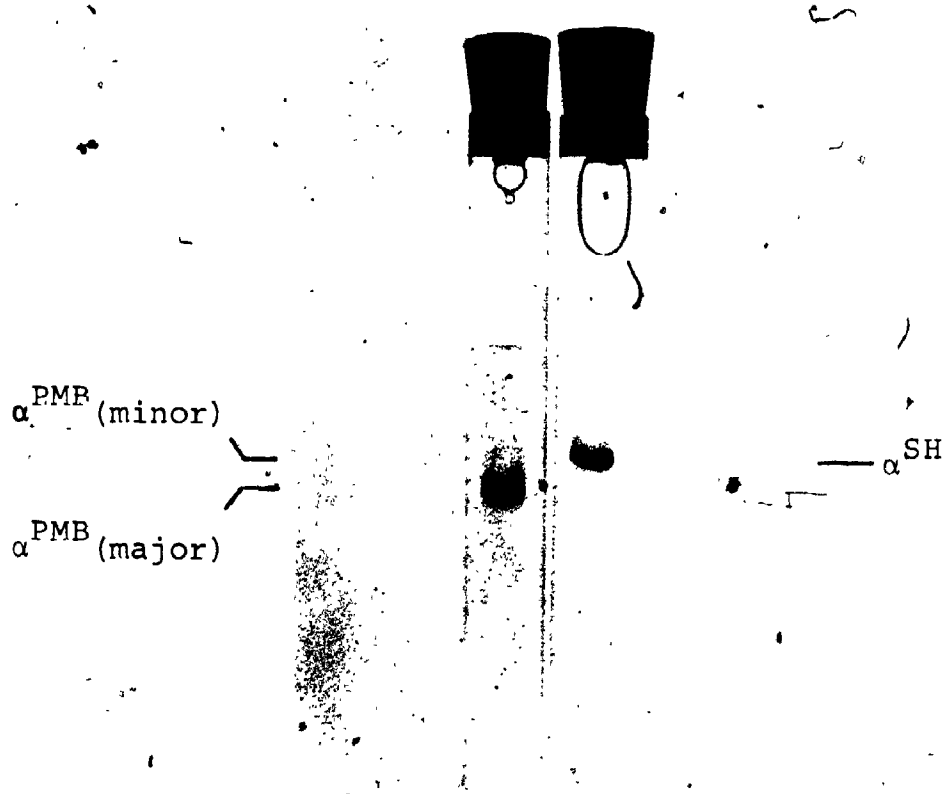
Figure 35: Polyacrylamide gel analysis of α^{SH} and α^{PMB} subunit purity.

Left: α^{PMB}

Right: α^{SH}

α^{SH} consisted of a single band

α^{PMB} possessed one major and one minor component


α^{PMB}(minor)
α^{PMB}(major)
α^{SH}

α^{SH} as expected, and a minor component with mobility similar to that of α^{SH}. Their relative amounts were approximately 83% and 17% as determined by gel band densitometry. The possibility of spontaneous loss of the PMB group from some of the α^{PMB} monomers during preparation resulting in conversion to α^{SH} was investigated by PCMB titration. However, no sulfhydryl groups were detected in a 1×10^{-2} mM solution of α^{PMB}. Other possible explanations included partial denaturation during preparation, storage or electrophoresis, as well as rapid dimerization coupled with slow breakdown:

$$\alpha^{PMB} \underset{\text{slow}}{\overset{\text{fast}}{\rightleftharpoons}} \alpha^{PMB}$$

Any suggestion of the minor component being the contaminant remaining after the DEAE-Sephadex step can be discounted since the α^{PMB} sample used for electrophoresis was G-75 Sephadex purified and proven to be free of the ability to bind strongly to haptoglobin. The α^{PMB} contaminant was, on the other hand, capable of formation of stable complex with haptoglobin. This question was not resolved and therefore any conclusions made from experiments involving α^{PMB} subunits must be carefully weighed.

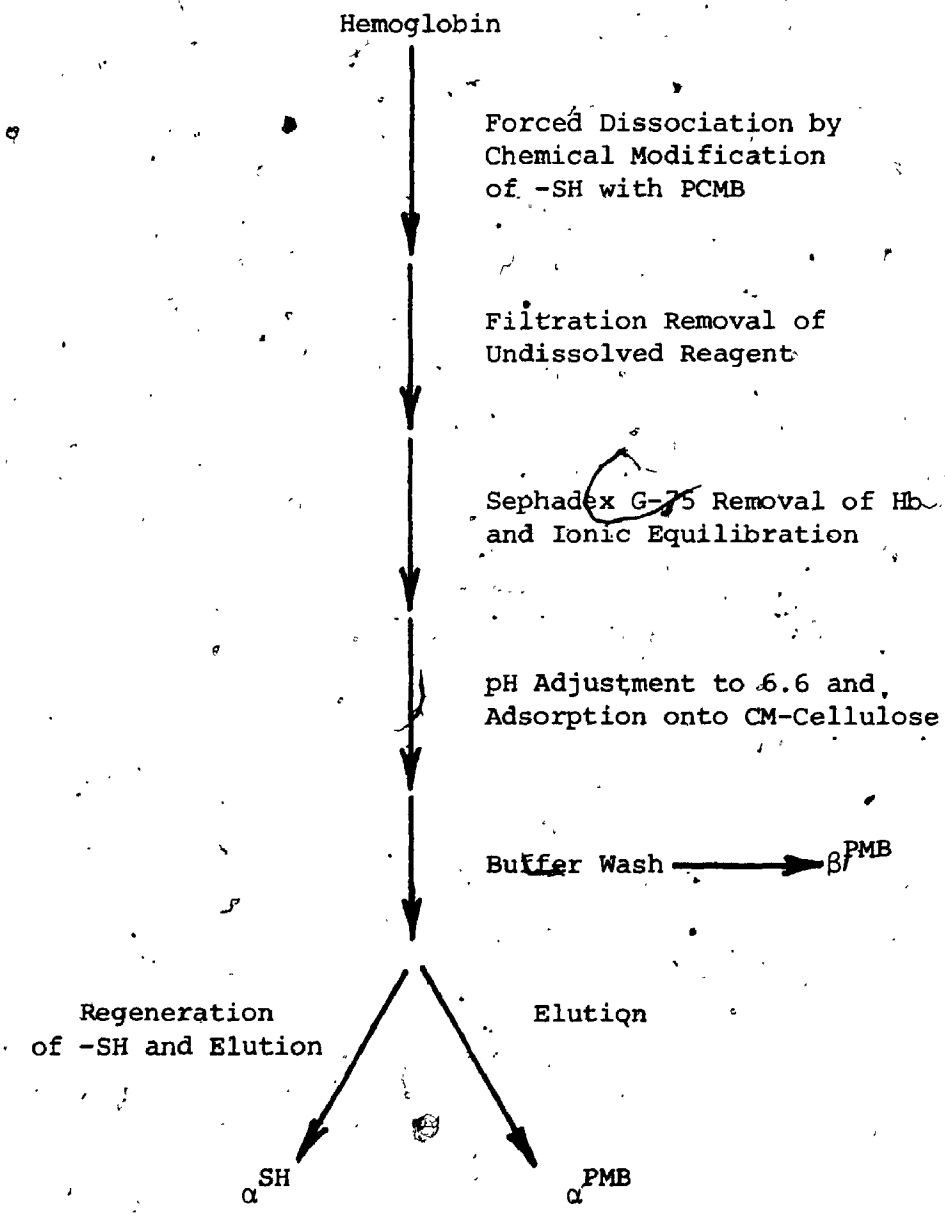
Alternate Method of Subunit Preparation

In terms of effective yield and concentration of α^{SH} isolated, the necessity of a final gel filtration step being added to the method Geraci et al (1969) is unfortunate. Not only does it involve a greater than ten-fold dilution, but much of the leading edge of the eluted α^{SH} is not useful because of trace amounts of contaminant while a considerable amount of the pure material in the trailing edge is too dilute for some purposes. This suggests the need of a more efficient method to produce higher concentrations without resorting to ultrafiltration or other means of concentration which in this case are of dubious advisability. A proposal of such a method is presented in Figure 36.

The reaction of stock hemoglobin with PCMB is unchanged from that described earlier, as is the filtration removal of undissolved reagent. The next step, G-75 chromatography in 10 millimolar phosphate - 2×10^{-5} molar EDTA pH 8.0, performs all the ionic adjustments of the G-25 column it replaces, and removes the partially and unreacted hemoglobin. It also reduces the ionic strength in anticipation of the CM-cellulose step to follow.

The DEAE chromatography in the method of Geraci et al (1969) was designed to separate the α^{PMB} subunits from the other two components present, namely hemoglobin and β^{PMB}. In the proposed method, removal of the

Figure 36: Proposed method of hemoglobin α subunit preparation.


Hemoglobin
Forced Dissociation by Chemical Modification of -SH with PCMB
Filtration Removal of Undissolved Reagent
Sephadex G-75 Removal of Hb and Ionic Equilibration
pH Adjustment to 6.6 and Adsorption onto CM-Cellulose
Buffer Wash
β^{PMB}
Regeneration of -SH and Elution
Elution
α^{SH}
α^{PMB}

hemoglobin is by the G-75 column while β^{PMB} are removed (and easily isolated) by their lack of binding to CM-Cellulose at pH 6.6.

Regeneration of sulfhydryl with β-mercaptoethanol is unchanged. It should be noted that the final step is now one which enhances concentration by the binding of protein in dilute solution followed by elution in a small volume while in the previous method it was gel filtration, a source of great dilution. Therefore the proposed procedure is at once less complex and more suited for higher concentration and yield.

Tests of the method are of course necessary. G-75 column chromatography, amino acid analysis, and polyacrylamide gel electrophoresis should determine purity of the subunits produced. Success, or the lack of it, rests upon the complete separation of hemoglobin from the α^{PMB} and β^{PMB} subunits. This in turn depends upon the effectiveness of the G-75 column step and the absence of sulfhydryl regeneration in the monomer fraction. In view of the profile in Figure 32 and the great stability of the mercaptide formed by PCMB with cysteine ($K_{DISS} = 10^{-15.7}$; Simpson, 1961), optimism is not unfounded. However, if PMB loss seems to occur even to a slight degree, a subsaturating amount of the reagent, perhaps stabilized by pyrophosphate (Muftic, 1970), can be added to the G-75 column buffer. Another option is the similar use of

p-chloromercuriphenyl sulfonate which is both more soluble and stable in an aqueous medium (Velock, 1953).

Due to an unfortunate lack of time, this new procedure was not investigated.

BINDING OF α SUBUNITS TO HAPTOGLOBIN

Preliminary Considerations

Having established the purity and quality of both haptoglobin and α subunit preparations, there remained several factors to be examined before binding studies could be undertaken. One of these was choice of wavelength to monitor absorbance. A second was the corresponding extinction values of α subunits.

A useful advantage embodied in these studies is the ability to select the detection of both haptoglobin and α subunits at 280 nm or just the latter in the visible. Spectophotometers available were reliable according to manufacturer's specifications in the vicinity of 280 nm of the ultraviolet and across the entire visible spectrum. One limitation was that the photoelectric scanner of the Model E ultracentrifuge was not recommended for use above 440 nm. Therefore using a carefully wavelength calibrated scan of the α subunits (Figure 34) it was observed that 413 nm for low and 3[illegible]8 nm for high protein concentrations were most suitable. No measurements were made in the ultraviolet below 300 nm.

The concentration of α subunit solutions was to be

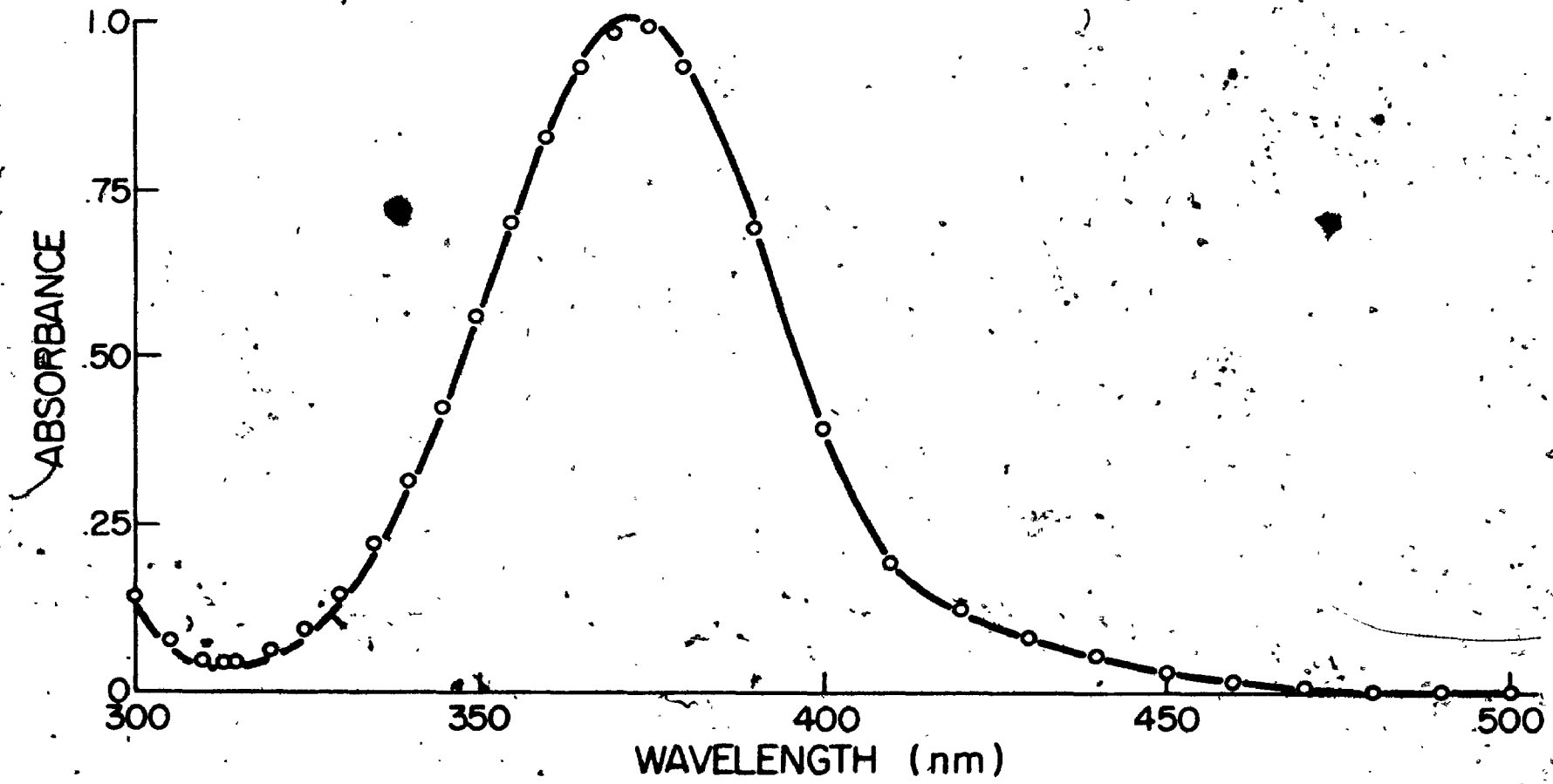
determined spectrophotometrically using Beer's Law. However the extinction coefficient at the chosen wavelengths was unknown. The only values found in the literature of α^{SH} were $E^{1\%}_{1\ cm}$ = 8.5 at 540 nm (Chiancone et al, 1968) and ε_{540} = 14.2 x 10^3 M^{-1} cm^{-1} for the carboxy form (Chiancone et al, 1966). In the case of α^{PMB}, DeBruin and Bucci (1971) have reported a value of ε = 14.0 x 10^3 M^{-1} cm^{-1} at 540 nm as the carboxy form. The Merck Index (1968) quotes ε for various forms of hemoglobin but none at 368 nm. In view of this, and the fact that it was not established that the constants of hemoglobin subunits were identical, the extinction coeffient of α^{SH} at 368 and 413 nm was determined in two ways, by a spectral approach and by amino acid analysis.

After precise baseline adjustment of the Cary 14 and 15 scanning spectrophotometers, the wavelength-absorbance characteristics of the instruments were verified with an alkaline potassium chromate standard according to the method of Haupt (1952). Figure 37 shows the excellent agreement between observed and reported profiles. The visible spectrum of α^{SH} subunits in PO_4 - EDTA was scanned and ε_{368} determined based on relative absorbance at 368 and 413 nm, and a 413 nm hemoglobin millimolar extinction coefficient of 125 (Merck Index 1968). The two values obtained, 21.7 and 22.4, were averaged to 22.0.

Figure 37: Comparison of the observed and reported spectrum of alkaline potassium chromate standard solution.

Solid Line: Observed spectrum on CARY 14

Open circles: Reported values (Haupt, 1952)


1.0
.75
.50
.25
0
ABSORBANCE
300
350
400
450
500
WAVELENGTH (nm)

The amino acid analysis method, involving no ε assumption, confirmed the use of a hemoglobin extinction coefficient as that of the isolated α subunits, as well as the value of ε_{368} which resulted. Closely agreeing absorbances of a solution of α^{SH} subunits in PO_4 - EDTA were measured at 368 and 413 nm on both the Cary 14 and 15 instruments and then averaged. An aliquot of this solution was then hydrolyzed and prepared for analysis with careful attention paid to volume. The number of micromoles of each amino acid detected was divided by the respective number in the known composition and these values averaged to give the best estimate of moles of protein analyzed. After correction for dilution, filtration loss, and volume changes upon dissolution in analyzer buffer, millimolar extinction values of 120 at 413 nm and 21.2 at 368 nm were calculated using Beer's Law. Table 5 lists the ε values as estimated by the spectral method and compares them with those from other sources where available. This ε_{368} was considered to be the most accurate and was therefore used, when required, in all experiments to follow.

A third pre-requisite of the study of haptoglobin α subunit interaction was the definition of the limits of pH beyond which conformational change, as detected by spectral modification, was known to occur.

A solution of α^{SH} subunits in PO_4 - EDTA was titrated with 0.1 N HCl at room temperature with gentle stirring.

Table 5: Extinction coefficient values of α^{SH} in PO_4-EDTA determined spectrophotometrically. Comparison is made with the literature where available.

Spectral Description	Wavelength (nm)	ε^{*}_{α} ($mM^{-1}cm^{-1}$)	$\varepsilon^{**}_{\alpha}$ ($mM^{-1}cm^{-1}$)
Peak	576	15.1	15.1 16.2
Trough	560	8.26	——
Peak	540	14.2	14.2 15.3
Plateau	510	5.41	——
Peak	413	125.***	125. 128.
Trough	368	22.0	——
Peak	343	27.3	——

* Cary Recording Spectrophotometer

** Merck Index

*** Assumed

Aliquots were removed and their visible spectrum immediately scanned on the Cary 14. Figure 38 presents the spectra observed. It is evident that no significant change occurs above pH 5.0 but there are apparently slight changes in extinction coefficient particularly in the Soret region since maximum absorbance at 413 nm does not parallel the gradual decrease in subunit concentration and pH as the titration progressed. Extensive changes appeared as the pH was decreased further, with the eventual acquisition of the acid spectrum of heme. Figure 39 shows the difference spectrum obtained with α subunits at pH 6.04 as reference and at pH 3.01 as sample. This graphically illustrates the disappearance of the Soret band and the transition from a trough to a peak in the region of 368 nm. Adjustment of the pH to 8.4 with 0.3 N NaOH resulted in only partial recovery of the native spectrum. This and a slight turbidity indicated that the denaturation was to a considerable degree irreversible at least in the half hour time span allowed for renaturation.

Thus in the acidic region of the pH scale, the presence of or large changes in the binding of α to Hp can be studied as low as pH 5.0 but accurate calculation of α concentration and number bound to Hp cannot be made due to variation of extinction coefficient. As an extension of the hemoglobin renaturation work of Sasazuki et al (1974) one could suggest, in passing, the study of the acid

Figure 38: Absorption spectrum of the α^{SH} subunit in the 300 nm to 500 nm range as a function of solution acidity. The respective spectrum number and pH is indicated.

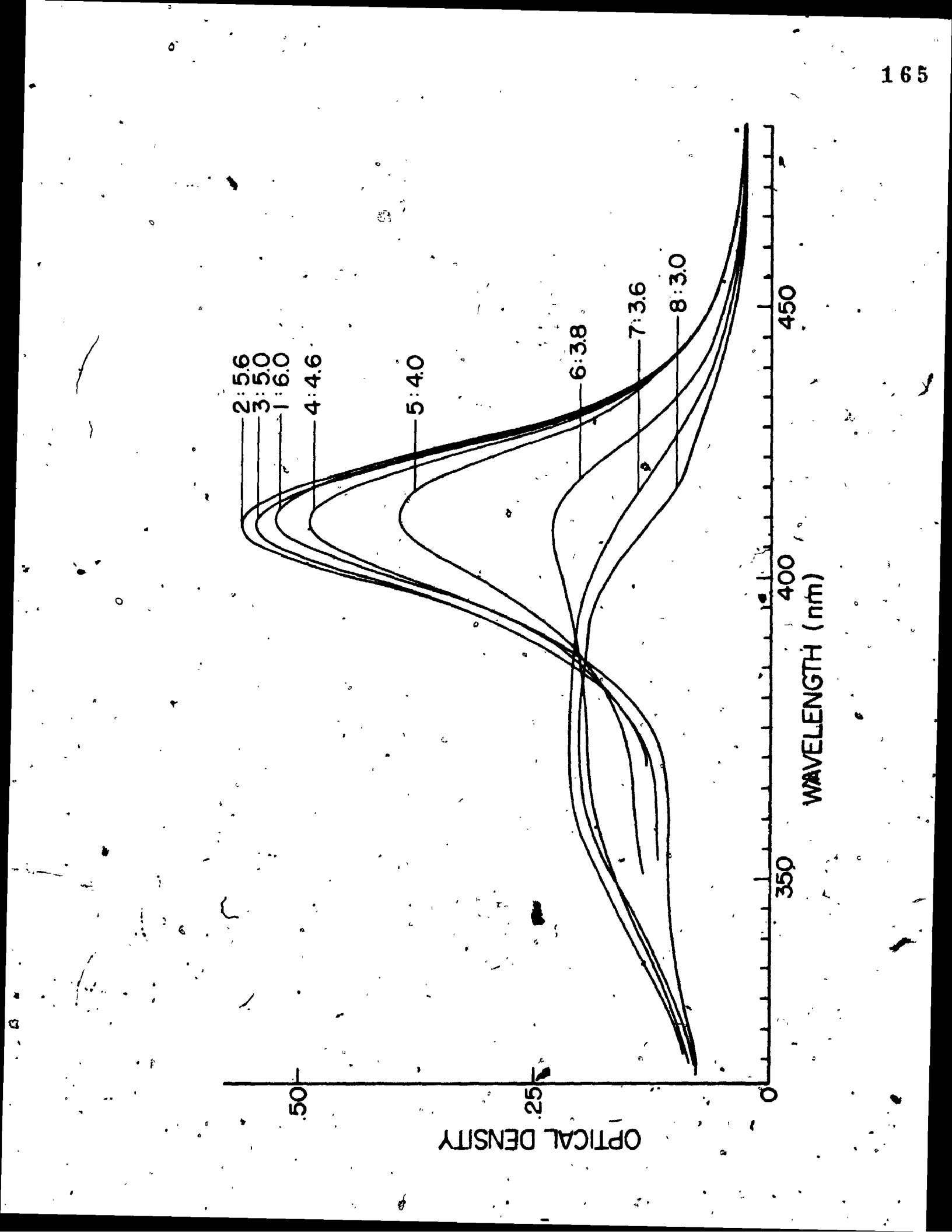
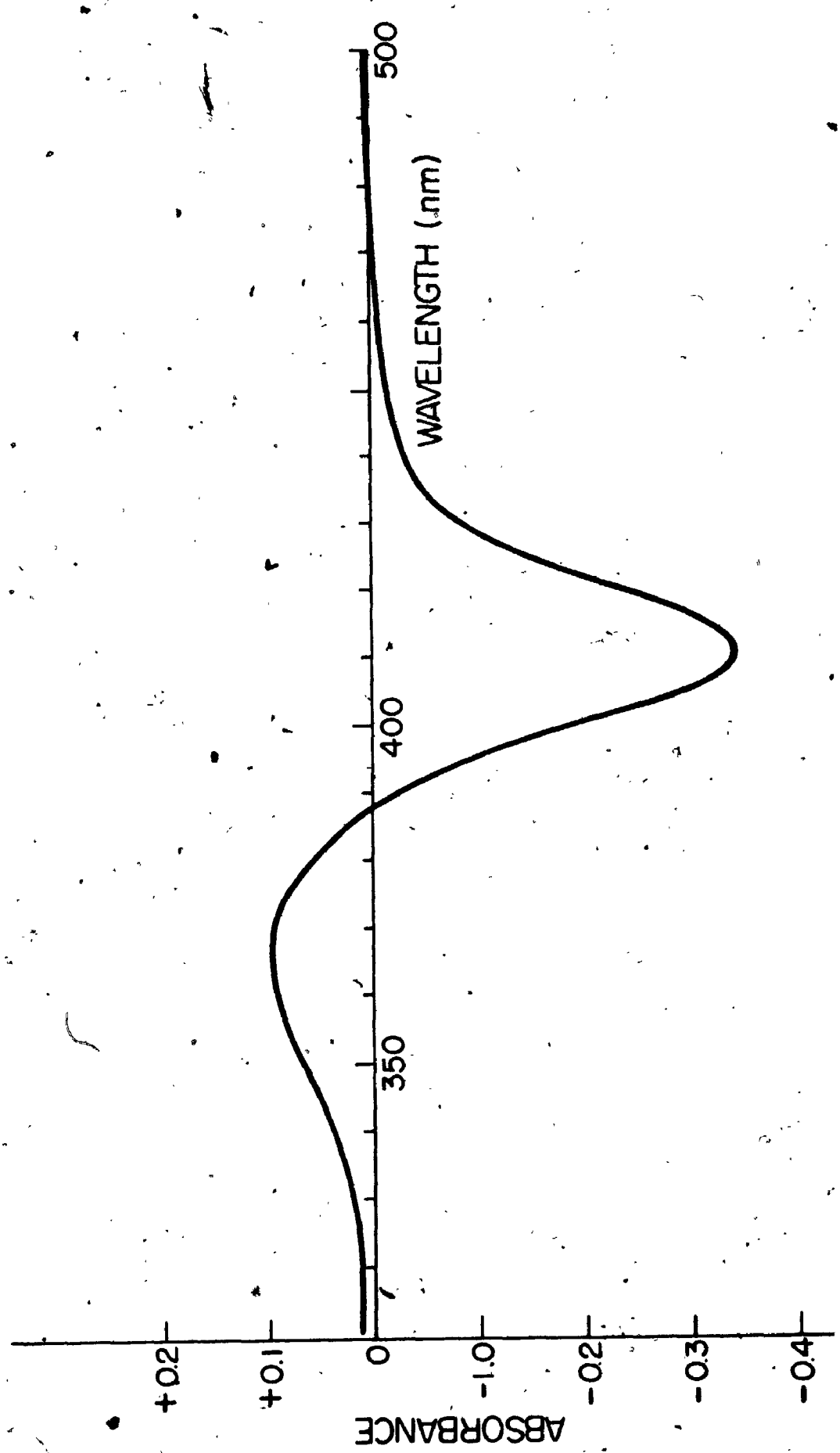

OPTICAL DENSITY
50
25
0
350
400
450
WAVELENGTH (nm)
2:5.6
3:5.0
1:6.0
4:4.6
5:4.0
6:3.8
7:3.6
8:3.0

Figure 39: Difference spectrum of the α^{SH} subunit near neutrality (pH 6.04) as reference and in acid conditions (pH 3.01) as sample.


WAVELENGTH (nm)
500
400
350
ABSORBANCE
+0.2
+0.1
0
-1.0
-0.2
-0.3
-0.4

denaturation state(s) of α^{SH} and the effect of a return to neutrality as well as the presence of haptoglobin on the rate and extent of reversal of this process.

The other end of the pH scale was also investigated, with conditions of 0.1 M sodium borate - 2×10^{-5} M EDTA pH 9.5 (borate - EDTA) being of particular interest as they were to be used later for crosslinking studies with a bifunctional reagent. After titration of α^{SH} to these conditions (with 0.2 M borate, pH 9.5, and 0.1 N NaOH) and incubation at room temperature for two hours, the visible spectrum was scanned and superimposed on that of a similar dilution of α^{SH} in PO_4 - EDTA (Figure 40). Spectral change was so slight as to be barely detectable. After 24 hours, the spectrum was seen to have decreased in intensity somewhat without any wavelength shift. As presented in Figure 41, increasing the pH to 10.5 had a similar effect, while above pH 11 drastic changes became evident with the spectrum beginning to approach that of heme. It was therefore concluded that although heme loss and possible extensive denaturation occurred above pH 11, no conformational change in α^{SH} was spectrally detectable during their exposure to pH 9.5 for several hours.

With this, preliminary investigations were complete.

Figure 40: Spectrum comparison of the α^{SH} subunit in PO_4-EDTA (0.1 M phosphate, pH 7.5 and 2 X 10^{-5} M EDTA) and in Borate-EDTA (0.1 M borate, pH 9.5 and 2 X 10^{-5}M EDTA). The respective spectra are as indicated.

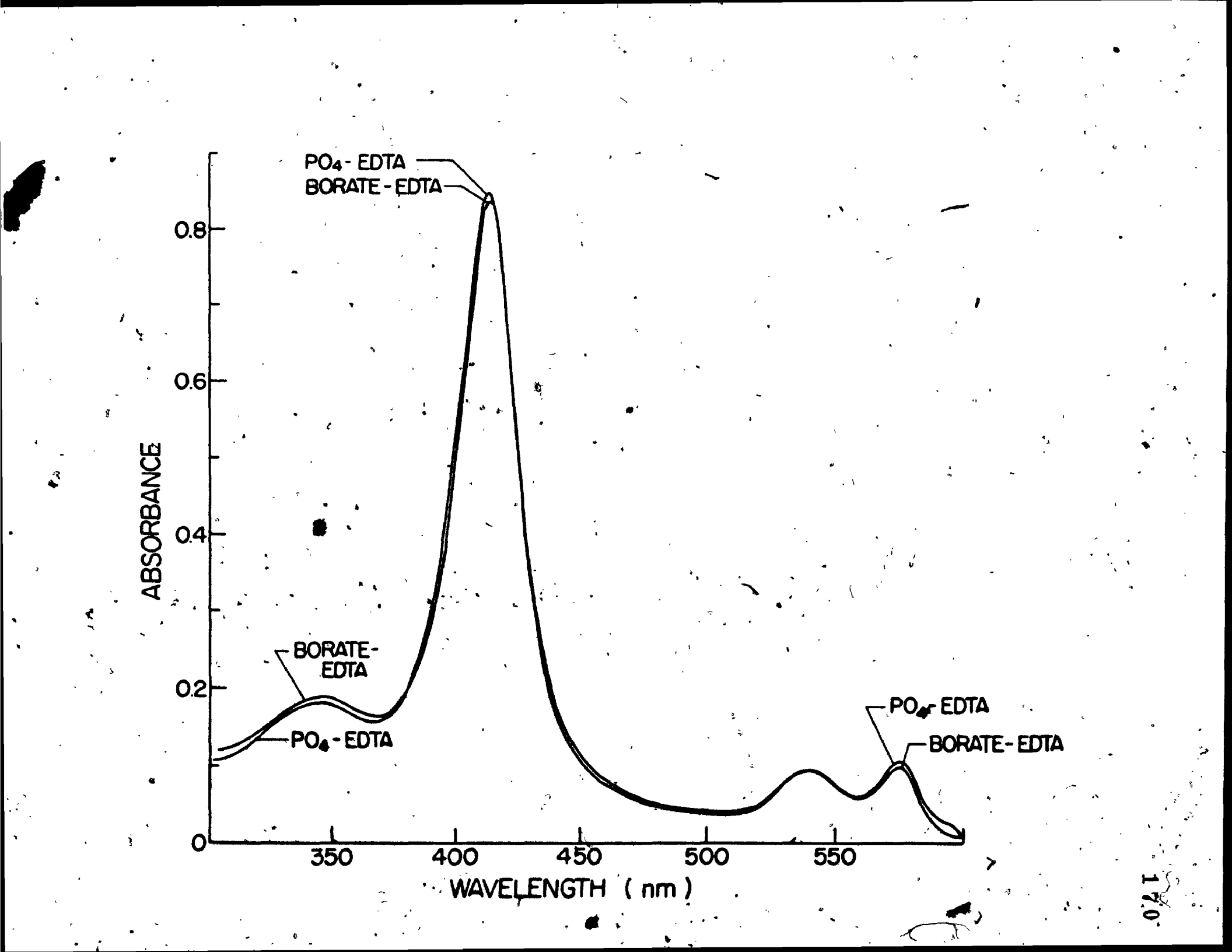
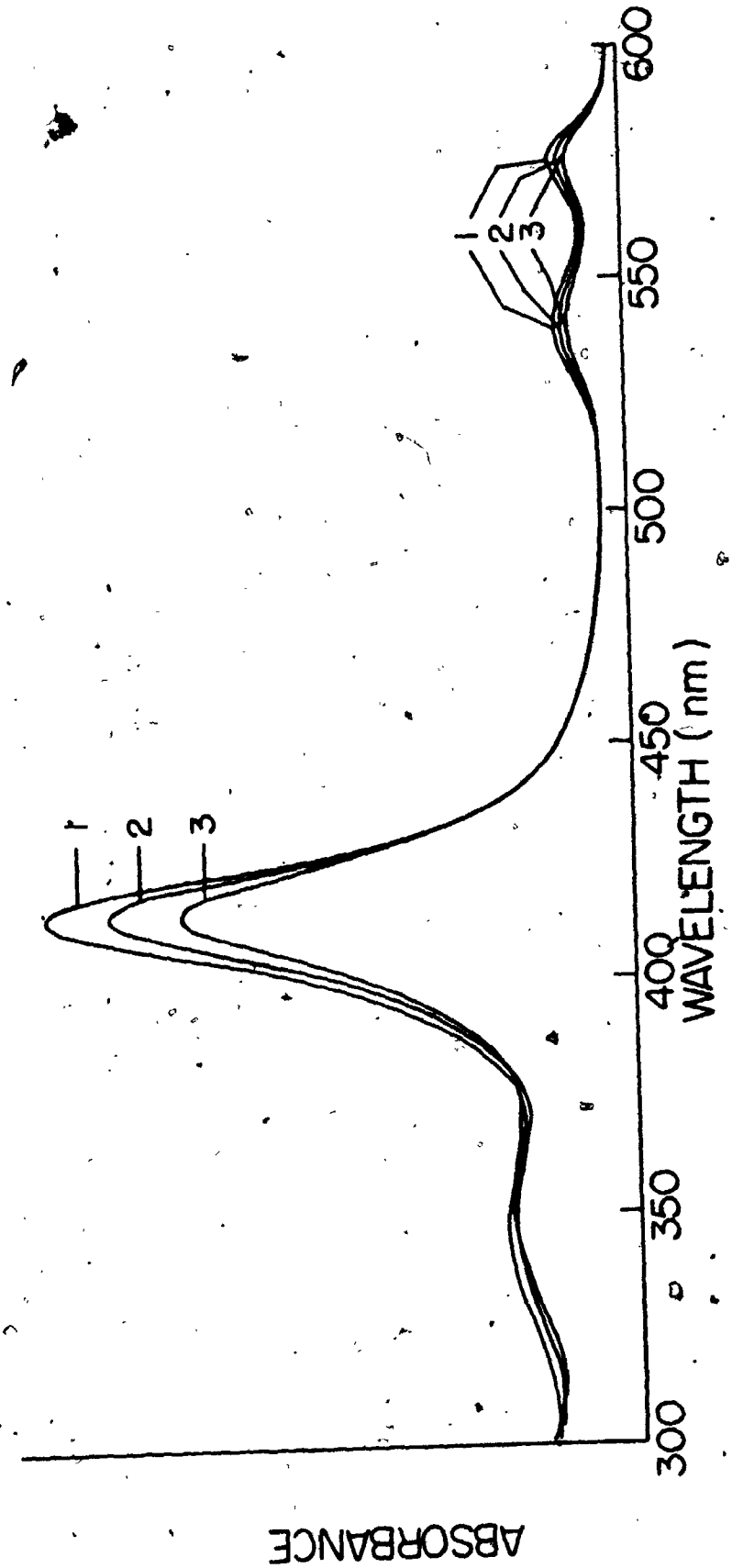

PO4 - EDTA
BORATE - EDTA
0.8
0.6
0.4
0.2
0
ABSORBANCE
BORATE-
EDTA
PO4 - EDTA
PO4 - EDTA
BORATE - EDTA
350
400
450
500
550
WAVELENGTH (nm)

Figure 41: Absorption spectrum of the α^{SH} subunit in the 300 nm to 600 nm range as a function of solution basicity. The respective spectrum number and pH are as follows:

Peak 1: pH 9.5

Peak 2: pH 10.0

Peak 3: pH 10.5


ABSORBANCE
300
350
400
450
500
550
600
WAVELENGTH (nm)
1
2
3
1
2
3

Isolation of Haptoglobin α Subunit Complex

Although Nagel and Ranney (1964) were unable to detect binding of isolated α subunits to haptoglobin by starch gel electrophoresis, Nagel and Gibson (1967). observed limited binding of α^{PMB} electrophoretically, as did Chiancone, Alfsen et al (1968). Unpublished results of Boyd (1971) indicated a biphasic saturation of haptoglobin with four subunits as seen by gel filtration, with degree of complexation affected by molar ratio, pH, total protein concentration, but not by time of incubation. The main aim of this present work was to confirm and extend these findings.

Before the discovery of a small amount of hemoglobin contamination in α subunit preparations, two complexes with haptoglobin were seen in poly acrylamide gels, but the molar ratio of α to Hp necessary for saturation varied with preparation. It was not understood how a system, which was evidently an equilibrium due to the requirement of molar ratios in excess of that of saturation, could produce complexes of sufficient stability to appear as well defined bands in PAGE.

Once the hemoglobin contaminant was removed from one preparation of α^{SH}, no complex band was seen with a ten-fold molar ratio of α to Hp, nor with a thirty or one hundred-fold ratio with another preparation. In an attempt to see some evidence of complex by decreasing the

available breakdown time, the electrophoresis was run very briefly (five milliamps per tube with tracking dye migration of one inch into separation gel). Still no complex band was apparent.

Gel filtration column chromatography was also unsuccessful in this regard despite variation of flow rate, column length, buffer concentration, pH, and Sephadex bead pore size. Early TLG studies with semi-pure haptoglobin indicated a Coomassie Blue-positive component slightly ahead of free haptoglobin which was interpreted as an α-Hp complex with surprisingly slow mobility. This was later identified as dimer albumin.

Previous reports of the isolation of complex did not present adequate proof of subunit purity. In addition, the presence of a contaminant below the detection level in a staining system would be amplified by the binding of the large haptoglobin molecule. These facts cast doubt on their validity. Binding forces were evidently much weaker than those of the hemoglobin haptoglobin system and an equilibrium was involved. In view of these considerations, and the work presented above, it was concluded that the study of the interaction of α subunits with haptoglobin must not entail the separation of any complex so formed from the unbound subunits. Study of the interaction was therefore undertaken in an α subunit environment.

αSH Binding to Haptoglobin in an α^{SH} Environment

Several techniques were considered for this purpose.

1. Ultrafiltration of a volume of α^{SH} plus Hp which was insignificant with respect to the solution volume in the ultrafiltration chamber was carried out using a membrane permeable only to hemoglobin monomer. This was to be followed by the complete recovery and the accurate determination of both concentration and volume of the filtrate as well as the solution remaining in the chamber. From these measurements, the free subunit concentration and hence the number bound per haptoglobin molecule was to be estimated. This method proved to be impractical.

2. The simultaneous dilution and ultrafiltration (diafiltration) method of Blatt et al (1968) appeared valuable but was not employed due to the nonreversible adsorption of α subunits to Diaflow membranes during the length of time required for this technique.

3. Equilibrium dialysis with α^{SH} as the dialyzable substrate had positive characteristics such as small amount of protein required and the opportunity to run a large number of cells concurrently (plexiglass micro cells of 250 ul sample chamber volume were available). Unfortunately none of the pore sizes of millipore filters used as dialysis membrane had suitable properties. Modification of pore size and shape of cellophane membranes for this purpose according to the method of Craig and Konigsberg

(1961) were considered somewhat uncertain and not attempted.

4. Thin layer gel filtration was tried. A sample of haptoglobin in a known excess of α subunits was applied to and chromatographed through a gel layer containing a uniform concentration of the subunits. The technique required the quantitative removal of gel beads and interstitial fluid followed by determination of absorbance at 280 and 413 mu. This removal proved to be too difficult.

5. Gel electrophoresis of haptoglobin through an acrylamide matrix polymerized in a homogeneous concentration of α^{SH} was considered but later discounted as being inappropriate for this system and fraught with experimental problems.

Sedimentation velocity experiments and supra-plateau studies proved to be more productive.

Quantitation of α^{SH} Binding to Haptoglobin

Plateau Analysis of Sedimentation Behavior

The application of the sedimentation velocity technique is particularly well suited for the investigation of α subunit binding to haptoglobin because of the considerable difference in the S value of the free subunit and that of Hp and its α^{SH} complex. Thus the complex reaches the cell bottom a sufficient time before the free subunit material to permit the estimate of concentration of the

latter from a well defined plateau. To illustrate, progressive scans beginning with that made at zero time are presented in Figure 42.

Haptoglobin sediments through a homogeneous environment of α and carries with it the complement of subunits corresponding to the position of equilibrium as defined by the molar ratio present. The other contributing parameters are maintained constant. All experiments were performed at near 6°C in identical ionic conditions (PO_4 - EDTA) and similar protein concentration.

Calculation of molar ratio in the samples and the number of subunits bound per molecule of haptoglobin were made as described in the Methods section. A test of the method using the hemoglobin-haptoglobin system yielded a value of 3.88 monomers bound per haptoglobin in good agreement with the known value of 4.0. The Hp-α^{SH} data is presented in Figure 43 and involves points determined on several dates and with different preparations. Despite considerable scatter, the degree of binding is seen to rise with but not as quickly as molar ratio. For example, at a molar ratio of two, there is a net binding of one α^{SH} per Hp. Near attainment of maximum binding is implied at a six-fold ratio. A least squares fit of the points beyond a ratio of seven indicates a line of slope 0.011 and y intercept of 2.18. Thus there is no significant upward trend and the maximum number bound, expressed as an integer,

Figure 42: Progressive sedimentation velocity ultracentrifugation scans of α^{SH} in the presence of Hp (3.9 fold molar ratio). Elapsed times are indicated. Traces at zero time and 2 hr 59 min were used for evaluation of plateau heights and hence the total and free α^{SH} concentration. Prior to centrifugation, the sample was 28.4 uM in α subunits and 7.4 uM in haptoglobin.

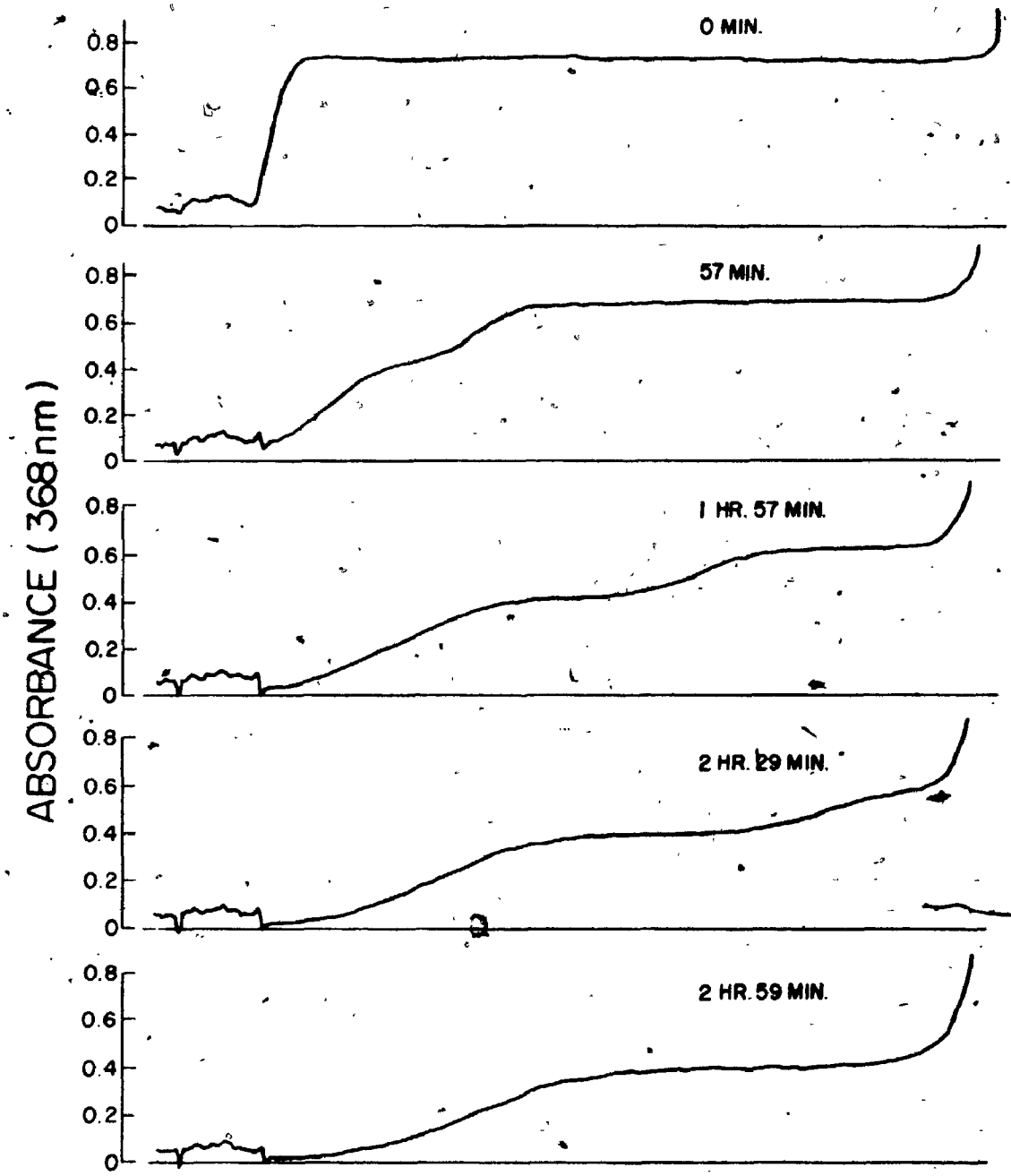
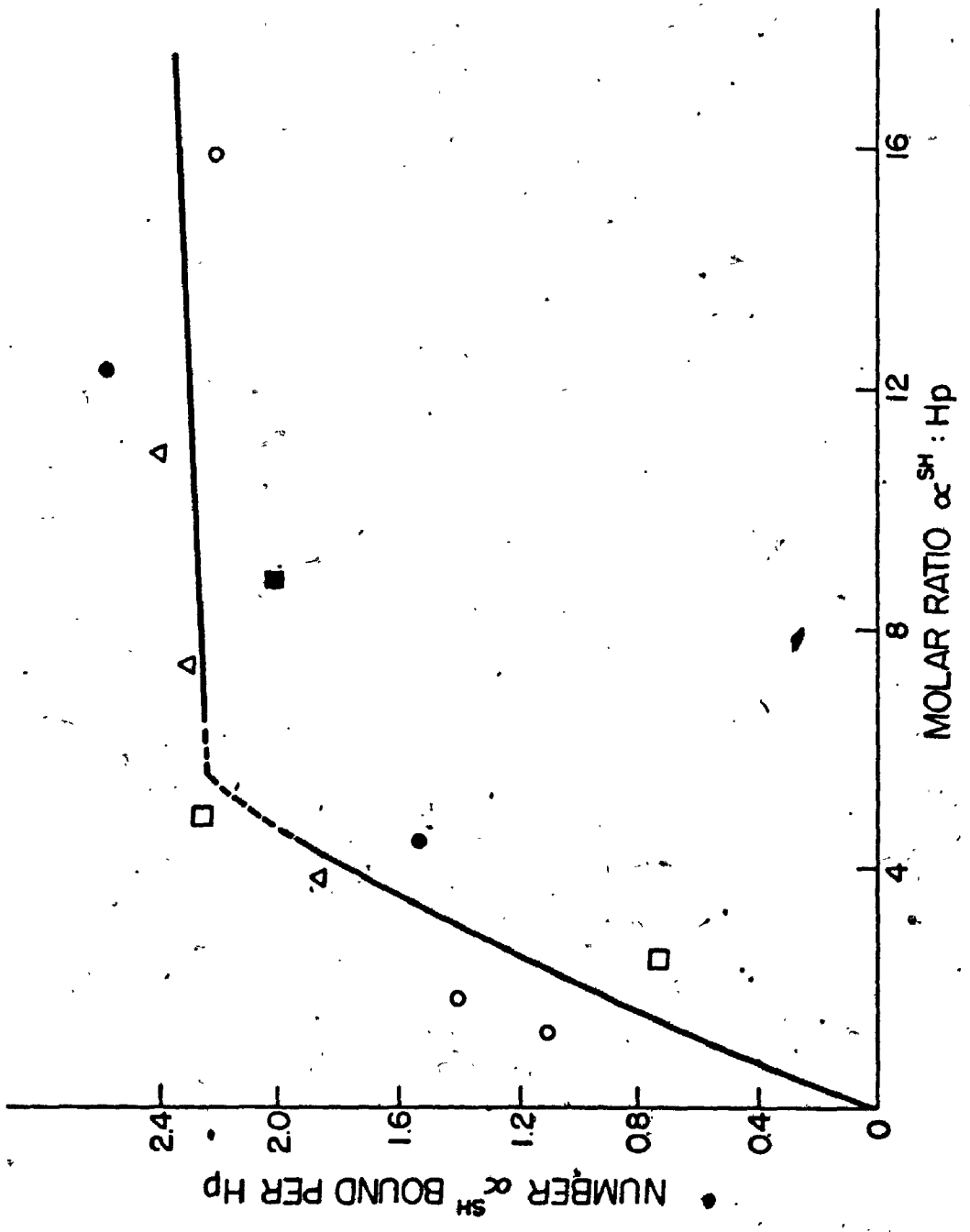

ABSORBANCE (368nm)
0 MIN.
57 MIN.
1 HR. 57 MIN.
2 HR. 29 MIN.
2 HR. 59 MIN.
0.8
0.6
0.4
0.2
0

Figure 43: Binding of α^{SH} to haptoglobin as a function of the α:Hp molar ratio as determined by sedimentation velocity ultracentrifugation plateau analysis. Open and closed circles (o,●) denote values obtained with a single preparation but at different times while other symbols (□,■,△) signify those obtained with different preparations.


NUMBER α^{SH} BOUND PER Hp
0
0.4
0.8
1.2
1.6
2.0
2.4
MOLAR RATIO α^{SH} : Hp
4
8
12
16

is two. An equilibrium of the type

$$Hp + \alpha \underset{\leftarrow}{\overset{k_1}{\rightleftharpoons}} Hp\alpha$$

$$Hp\alpha + \alpha \underset{\leftarrow}{\overset{k_2}{\rightleftharpoons}} \alpha Hp\alpha$$

is indicated. On the basis of the classic Michaelis-Menten nature of the binding curve, the two equilibrium constants are similar in magnitude. In addition there is no detectable interaction between the two binding sites for the α subunit. These findings are consistent with those of Nagel and Gibson (1971).

The very slight positive slope beyond a seven-fold α:Hp molar ratio might be the product of insufficient data but if assumed to be real could be interpreted as a very weak interaction of α with β sites and/or a self-association of α monomers. Investigations of α:α interaction are presented later.

Supra Plateau Gel Filtration

The supra plateau technique provided further support of this reaction scheme. In the section devoted to Methods, the procedure and its aims are discussed. Briefly stated, complex of haptoglobin and the α subunit of hemoglobin migrates into and through a medium of constant α^{SH} composition and creates a supra plateau. The establishment of a supra plateau indicates a region of homogeneous Hp as well as free and bound α^{SH} concentration in which there is no net loss or gain of α^{SH} by haptoglobin as migration through the α^{SH} plateau continues. Attainment of this equilibrium is hastened by beginning with the

haptoglobin in conditions similar to those it will experience in the α^{SH} plateau and of course by the use of large sample volumes and a chromatographic column of appropriate Sephadex gel and length. The height of the supra plateau above the α^{SH} plateau upon which it is superimposed is a direct measure of the concentration of haptoglobin bound α^{SH}. The concentrations of haptoglobin as well as bound and total monomer in the supra plateau region being known, the number of α^{SH} bound per haptoglobin and the molar ratio of α:Hp present can be calculated. A theoretical supra plateau study profile is presented in Figure 44.

The control system of haptoglobin and hemoglobin was employed to verify the applicability of the technique. The apparent number of monomers bound per Hp was 4.19, in close agreement with the theoretical 4.0. When the haptoglobin and α subunit system was tested using a 7.4 molar ratio of α:Hp and experimental details very similar to those presented in Table 2, the apparent number of α bound per Hp was 1.76. The supra plateau study profile observed is presented in Figure 45 and the data in Table 6.

An increase in molar ratio results in an inherent decrease in accuracy due to limitations on total monomer concentration and the fact that the absorbance of bound α monomers more closely approaches the errors in plateau heights introduced for example by the spectrophotometer and recorder. However when the experiment was repeated

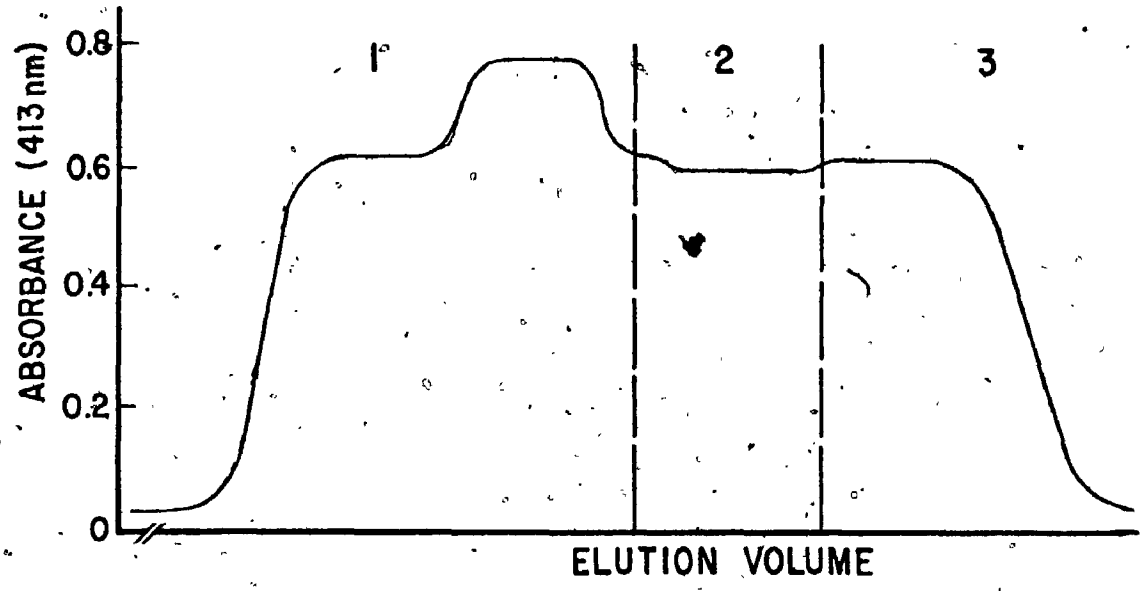
Figure 44: Theoretical supra plateau study profile. The regions resulting from the injected samples are numbered.

A plateau which originated in sample region two is seen superposed upon that plateau established by sample number one. A slight trough is seen in the wake of this migration. Region number three results from a chase sample of composition identical to that of sample number one.

Figure 45: Experimental supra plateau study profile involving α^{SH} and Hp.

The monomer concentration of samples number one and three was 4.7 μ M. Sample number two was 0.8 μ molar in Hp and 6.3 μ molar in α^{SH}. Therefore an initial α:Hp molar ratio of 7.9 was present.

The α:Hp molar ratio in the supra plateau of region number one, following chromatography, was 7.4.


ABSORBANCE (413 nm)
0.8
0.6
0.4
0.2
0
1
2
3
ELUTION VOLUME

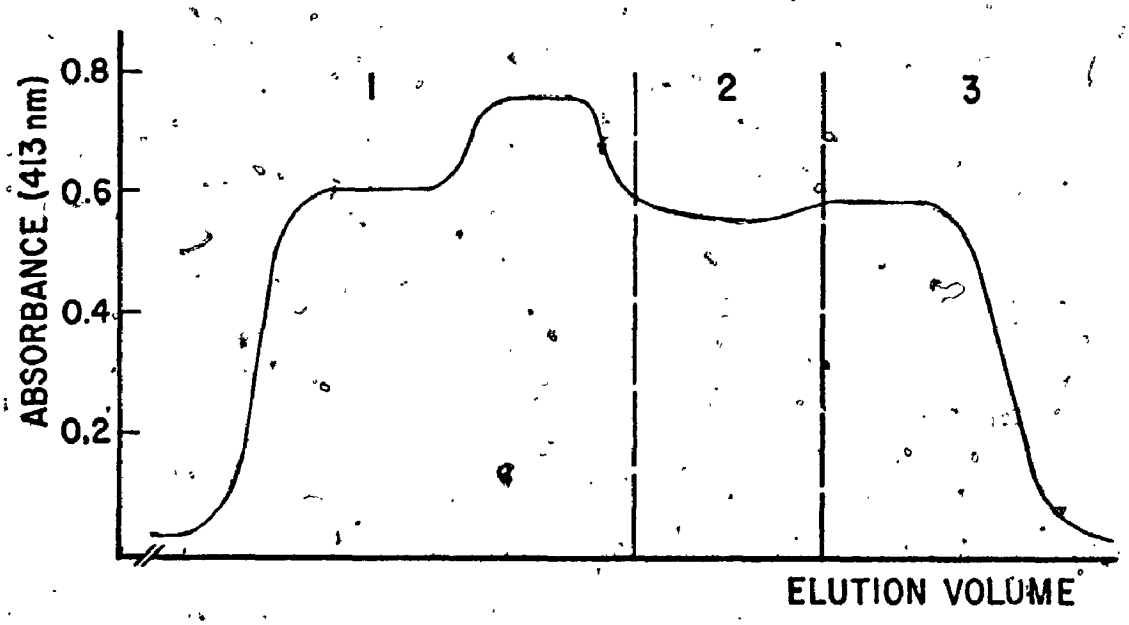

ABSORBANCE (413 nm)
0.8
0.6
0.4
0.2
1
2
3
ELUTION VOLUME

Table 6: Data resulting from supra plateau studies of hemoglobin and of its α-subunit with haptoglobin. Monomer to haptoglobin molar ratios were 9.7 and 7.4 respectively.

Table 7: Sedimentation coefficient of the complex formed by haptoglobin with hemoglobin and its α^{SH} subunit. Comparison is made with literature values of the former and of haptoglobin

Protein Bound by Haptoglobin	Molar Ratio Monomer/Hp	# Monomers Bound/Hp
$(\alpha\beta)_2$	9.7	4.19
α	7.4	1.76

Protein	Source of Data	$S_{20,w}$
HpHb	Literature	6.2 to 6.6
HpHb	Experimental	6.4
$Hp\alpha_n$	Experimental	4.5
Hp	Literature	4.1 to 4.3

with an α:Hp molar ratio of 12.5, the number bound per Hp still remained below two.

In the design of the haptoglobin-containing samples, it was assumed that haptoglobin would react stoichiometrically with one hemoglobin molecule or two α subunits. An excess was included such that the concentration of non-bound monomers would be similar to that of the first and third sample regions. Thus comparison provided a means of evaluating the accuracy of these assumptions. In all cases, the three regions approached colinearity.

Sedimentation Coefficient Determination

Sedimentation coefficient determination of the complex formed by haptoglobin with α subunits (molar ratio α:Hp of 8.9) required somewhat special photoelectric scan analysis. The complex to free α boundary was never well defined in that the complex and free α regions did not become horizontal before the complex piled up on the cell bottom. Thus the normal method of finding the boundary mid-point was not applicable. An alternate method was therefore devised. Estimates of the two plateau heights were made on more appropriate scans and found to be remarkably constant. They were then assumed applicable to those scans which best described the complex to free α boundary. Mid-point positions were measured and treated in the normal manner. Scans of a sample consisting of HpHb complex in excess Hb required similar analysis. The resulting LOG x, that is, LOG of the distance of the boundary to the centre

of rotation, versus Elapsed Time graphs are shown in Figures 46 and 47. The S values corrected to standard conditions are presented in Table 7. That of HbHp agrees well with the literature value (Chiancone, Alfsen et al, 1968) and is much greater than that of Hp-α complex indicating that Hp molecules in these conditions are certainly not saturated with four α subunits. The sedimentation rate of the faster moving boundary in the Hp-α subunit mixture is a reflection of the relative proportions of the forms in which Hp is present. The similarity of the observed S value with the sedimentation coefficient of free haptoglobin indicates that non-complexed Hp is the predominant species.

Discussion of α^{SH} binding to Haptoglobin

The question of how these findings relate to previous work with a view to resolution of apparent conflicts naturally arises. Three papers involve the study of Haptoglobin-α subunit interaction in an α environment.

The earliest, by Chiancone, Wittenberg et al (1966), detected the presence of complex, but no insight was gained as to its nature due to the formation of a "red precipitate" during ultracentrifugation, Chiancone,

Figure 46: Graph of LOG x versus elapsed time for the sedimentation coefficient determination of $Hp\alpha_n$ complex. The variable x denotes the distance between the sedimenting boundary and the centre of rotation.

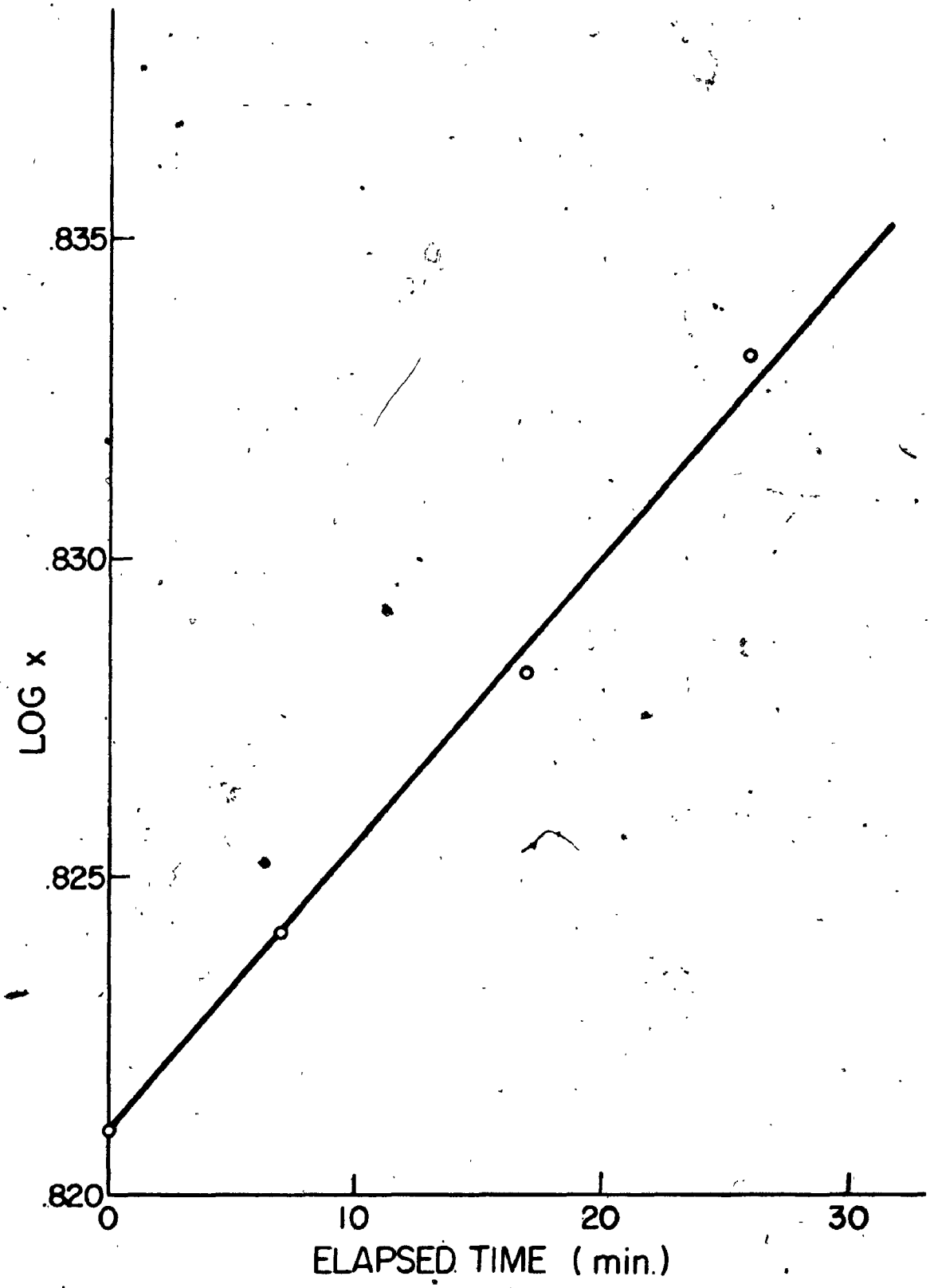
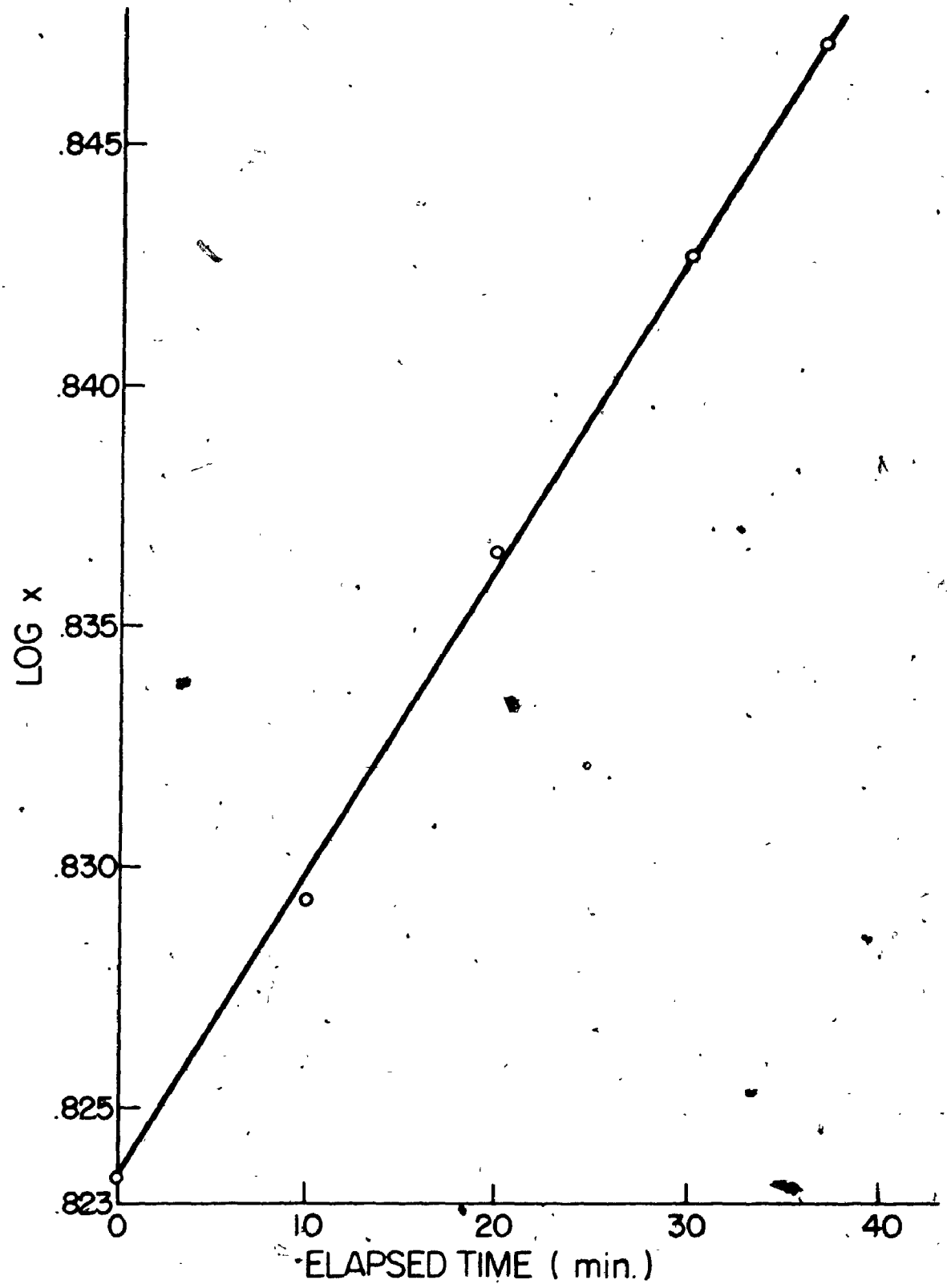

.835
.830
.825
.820
LOG x
0
10
20
30
ELAPSED TIME (min.)

Figure 47: Graph of LOG x versus elapsed time for the sedimentation coefficient determination of HpHb complex. The variable x denotes the distance between the sedimenting boundary and the centre of rotation.


.845
.840
.835
.830
.825
.823
LOG x
0
10
20
30
40
ELAPSED TIME (min.)

Alfsen et al (1968) presented a graph of $S_{20,w}$ versus molar ratio heme bound per molecule haptoglobin which indicated saturation as implied by an S value near that of HbHp complex at a four-fold molar ratio of α:Hp. However data later presented in the same publication which was intended to prove the displacement of α by Hb from the surface of Hp involved an S value for Hpα complex in a three-fold molar ratio which was clearly inconsistent with the first graph. In addition, fluorescence quenching curves were interpreted to prove the binding of four subunits per haptoglobin but the maximum quenching of Hp aromatic residues by α did not closely approach that seen with added hemoglobin. Thus the credibility of this report is questionable. The present findings support and confirm by alternate methods the most recent suggestion of Nagel and Gibson (1971) that haptoglobin binds two subunits. The formation of two β-specific sites which is said to accompany this binding is not here examined but the specificity for β of these secondary sites is virtually exclusive as indicated by the lack of interaction with α subunits at high molar ratios as seen in the ultracentrifuge.

All of the previous work in this field involved the use of human Hp 1-1 while the present work employs porcine Hp. However the physical, chemical and hemoglobin-binding similarities of the two (Fraser and Smith, 1971;

Black et al, 1970) make findings directly comparable.

In summation, the binding of human hemoglobin α^{SH} subunits to porcine haptoglobin involves an equilibrium of the type

$$Hp + \alpha \overset{K_1}{\rightleftharpoons} Hp\alpha$$

$$Hp\alpha + \alpha \overset{K_2}{\rightleftharpoons} \alpha Hp\alpha$$

Haptoglobin retains a full complement of two α^{SH} at a molar ratio α:Hp of approximately six. No significant further binding is detectable by the methods employed up to a molar ratio of fifteen. Thus interaction of α^{SH} with Hp β-specific sites is minimal. Support is given to the most recent contribution to the topic (Nagel and Gibson, 1971) and reasons put forward as to the source of inconsistency in the pertinent literature.

SELF-ASSOCIATION OF α-SUBUNITS

In the past, evidence has been presented (Ranney et al, 1965; Bucci et al, 1965) that α subunits exist primarily as monomers but do partake in limited association. The heterogeneous nature of the preparations employed coupled with the fact that denaturation commonly results in aggregation (leading to eventual precipitation) make the conclusions less than firm. The slight rise in α binding to Hp seen in the ultracentrifuge at very high α:Hp molar ratios described above prompted an investigation of this question of α self-association. The demonstrated

purity and stability of the subunits available implied that results obtained could be reported with confidence.

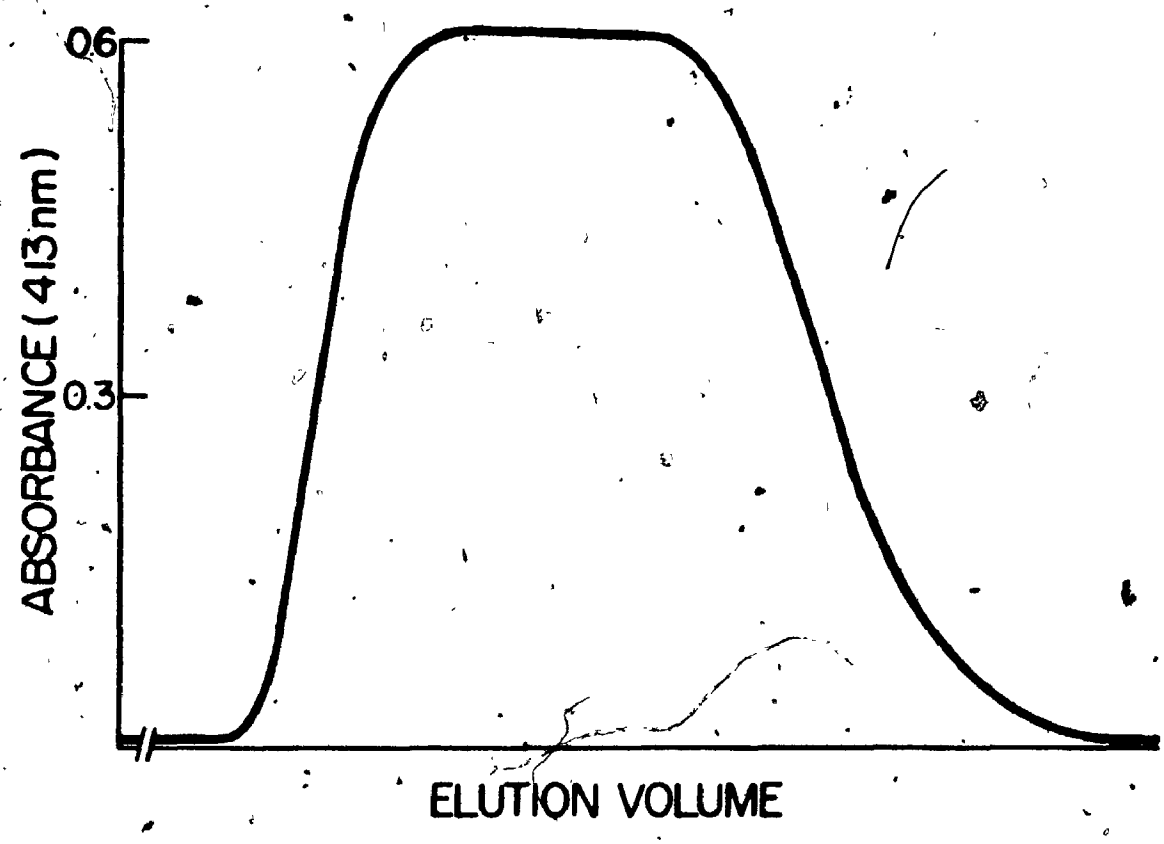
Gel Filtration Plateau Studies

The major technique chosen for this investigation was that of gel filtration edge studies used by Winzor and Scheraga (1963) to detect the association of α-chymotrypsin. Apparatus as outlined in Methods was designed to produce and record the elution profile of a large volume protein sample to permit comparison of the leading and trailing edges. Differences, which would imply association, could best be detected by plotting the absolute value of the first derivative to obtain a slope graph.

To determine whether the experimental system as constructed was of sufficient sensitivity to detect association, hemoglobin (413 nm) was used as a reference. The resulting profile and slope graph clearly confirmed this capability (Figure 48). As control proteins, myoglobin (409 nm) and cytochrome C (410 nm) showed no evidence of intermolecular interaction (Figure 49). (Spectrophotometer monitoring wavelengths are in parentheses.) In contrast, association of α^{PMB} was slight but consistently detectable while that of α^{SH} was more pronounced.

These observations cannot be attributed to the presence of hemoglobin since the elution profiles did not

Figure 48: Plateau study elution profile and resulting slope graph of hemoglobin in PO_4-EDTA. Monitoring wavelength was 413 nm.


0.6
0.3
ABSORBANCE (413nm)
ELUTION VOLUME

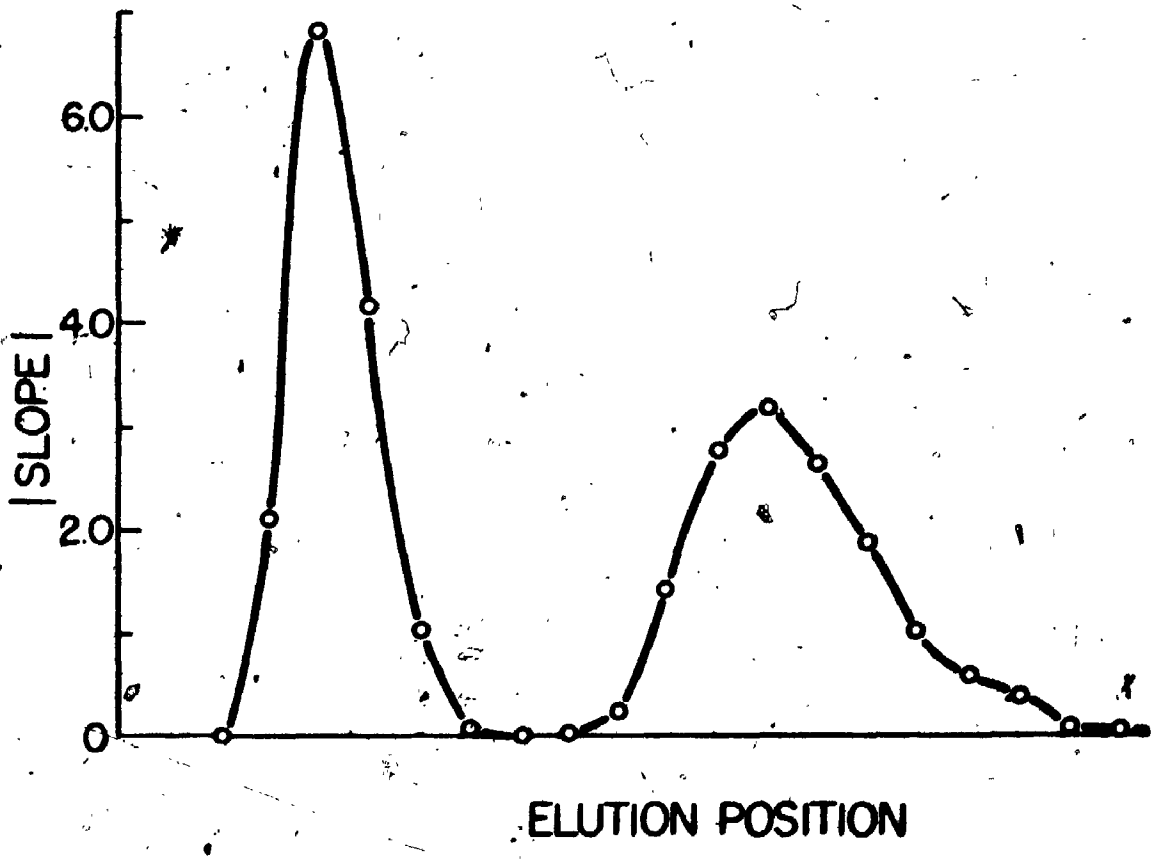
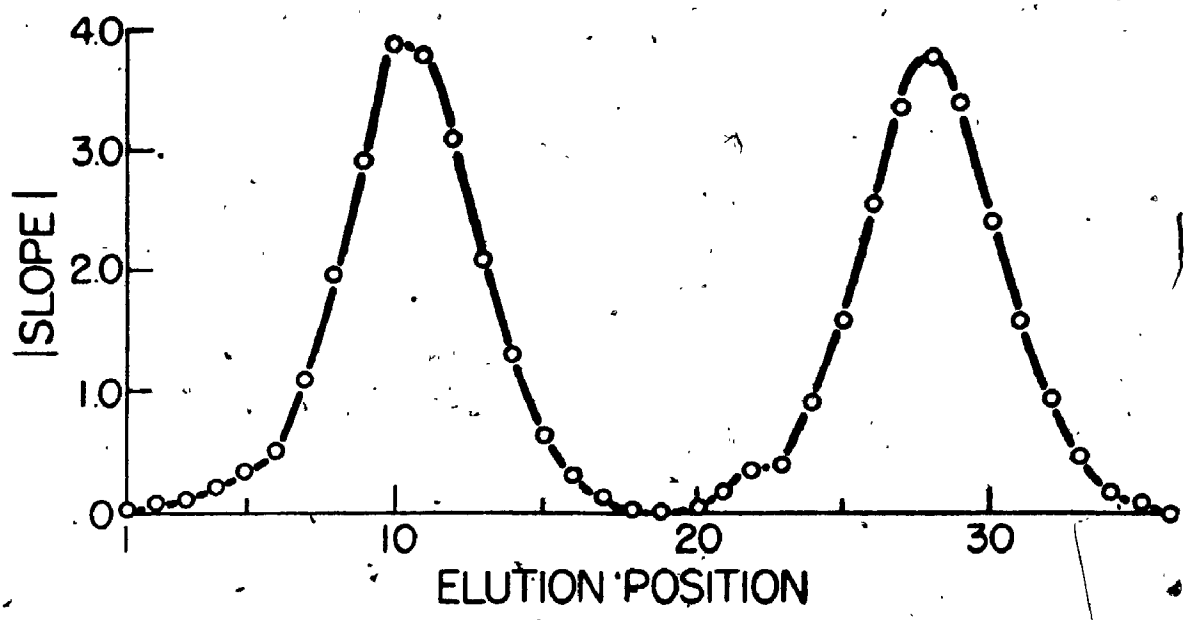
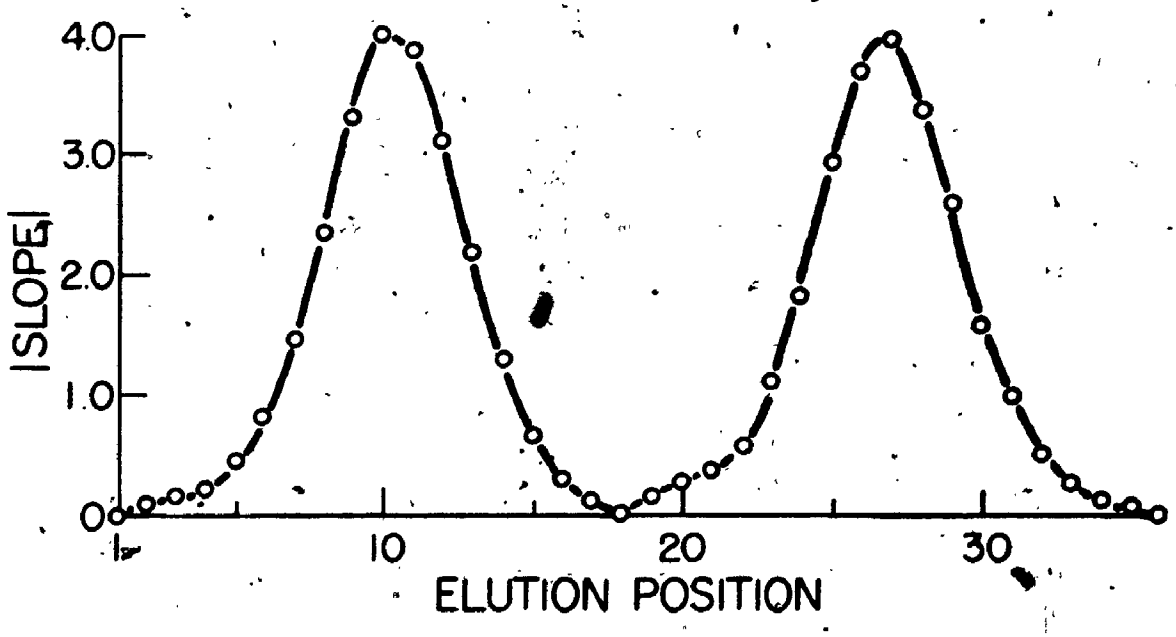

6.0
4.0
2.0
0
|SLOPE|
ELUTION POSITION

Figure 49: Plateau study slope graph of myoglobin (409 nm) and of cytochrome C (410 nm) in PO_4-EDTA. The wavelength at which the respective elutions were monitored is in parentheses. Differentiation of the elution profiles was begun at the first appearance of protein in the eluant.

Upper: Myoglobin

Lower: Cytochrome C


4.0
3.0
2.0
1.0
0
|SLOPE|
10
20
30
ELUTION POSITION

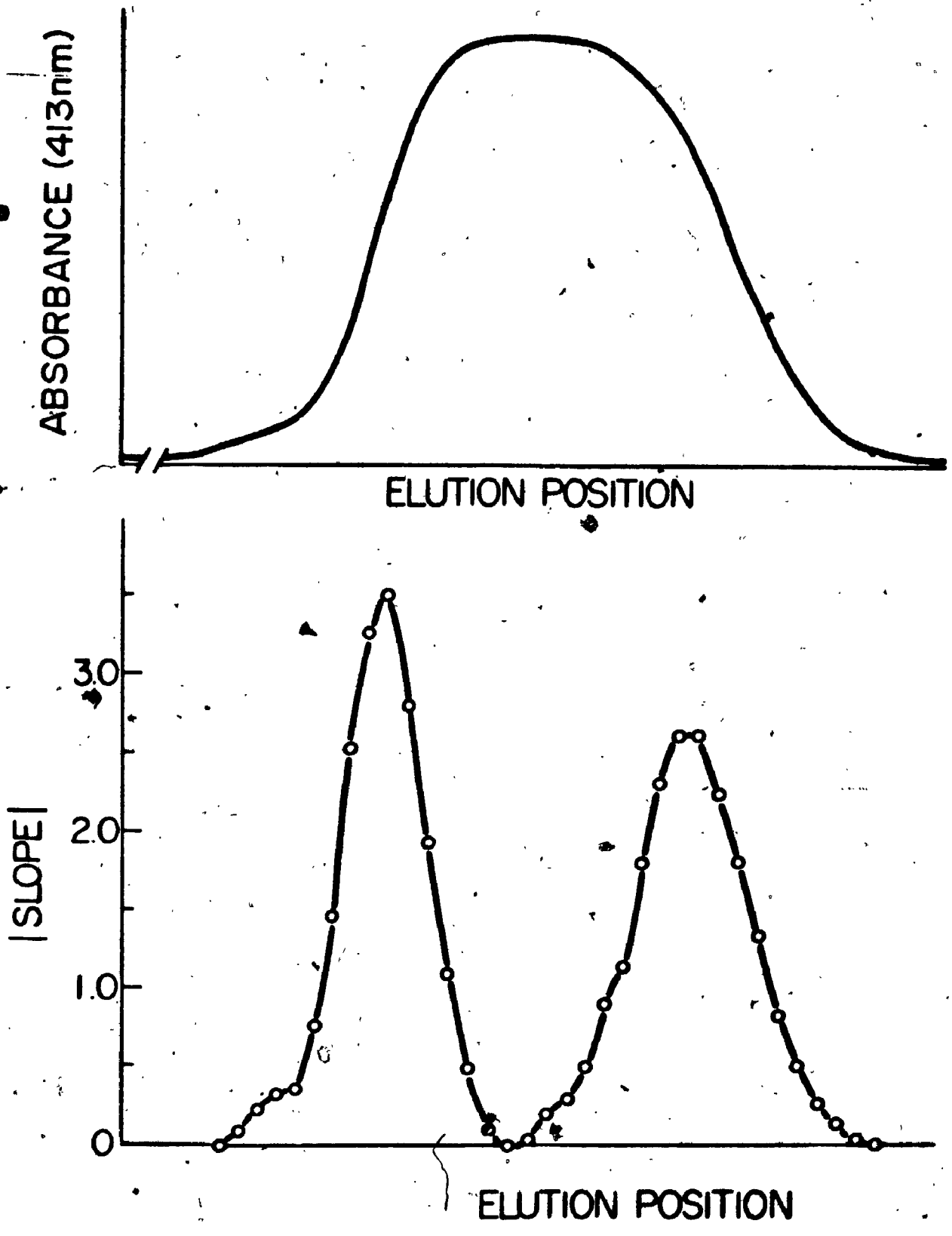

4.0
3.0
2.0
1.0
0
|SLOPE|
10
20
30
ELUTION POSITION

ressemble that of a prepared sample of 95% α · 5% Hb (Figure 50: note the leading edge shoulder and lack of a flat plateau region in the profile as well as the leading edge shoulders in the resulting slope graph). All α subunit materials were proven free of contaminant by the absence of stable complex upon the addition of haptoglobin as tested by the binding capacity method. In the absence of haptoglobin, the subunits chromatographed and sedimented as single symmetrical peaks, indicating no irreversible aggregation.

Association-dissociation phenomena by their very nature involve an equilibrium. Therefore the degree of association must be concentration dependent. A plateau study of α^{SH} monitored at 368 nm with sample six times as concentrated as that at 413 nm showed increased edge asymmetry. The slope graphs are compared in Figure 51. There was no wavelength within the range of the Beckman DB spectrophotometer at which α^{SH} possessed an extinction coefficient significantly greater than at 413 nm. This ruled out lowering the concentration to a point where perhaps no association would be detected.

An α^{SH} plateau study in PO_4 - EDTA after titration to pH 9.5 with 0.1 N NaOH showed a reduced degree of association and a barely detectable leading shoulder. This shoulder, thought to consist of non-reversibly aggregated metα^{SH} formed as a result of instability at this pH, was not a product of the mode of pH change as

Figure 50: Plateau study elution profile and resulting slope graph of a 95% α^{SH}. 5% Hb solution.


ABSORBANCE (413nm)
ELUTION POSITION
|SLOPE|
3.0
2.0
1.0
0
ELUTION POSITION

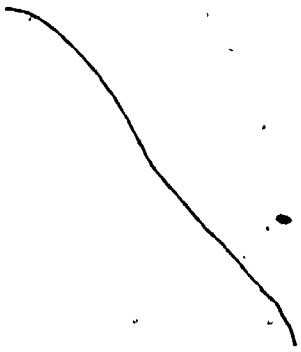
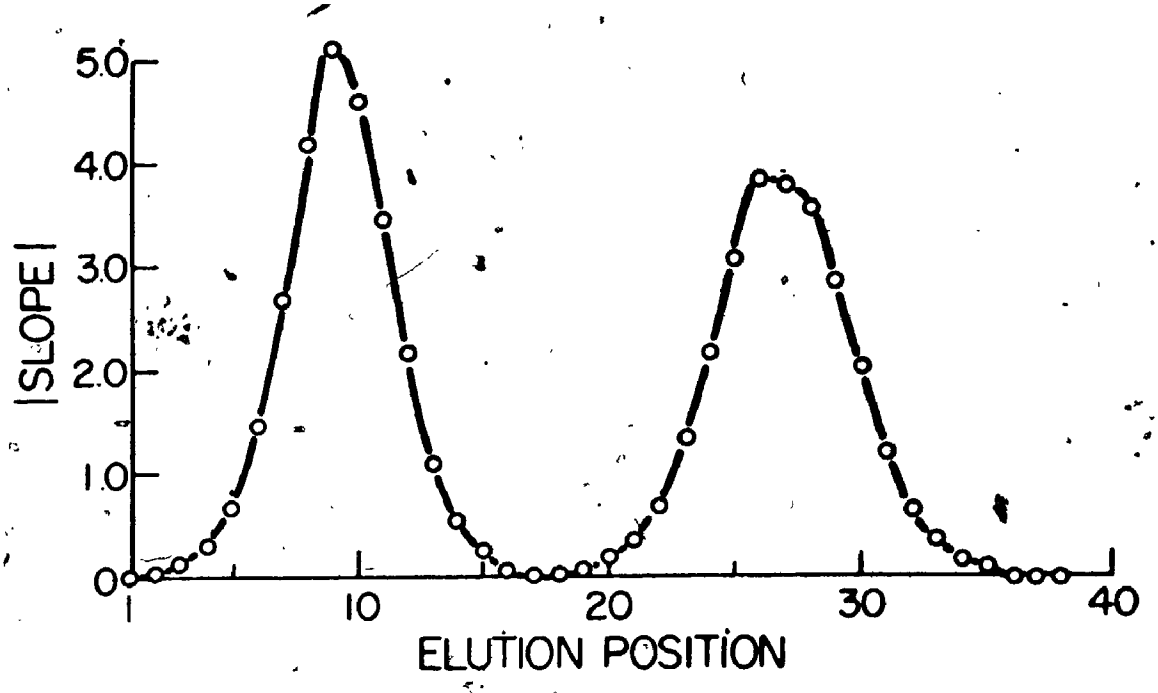
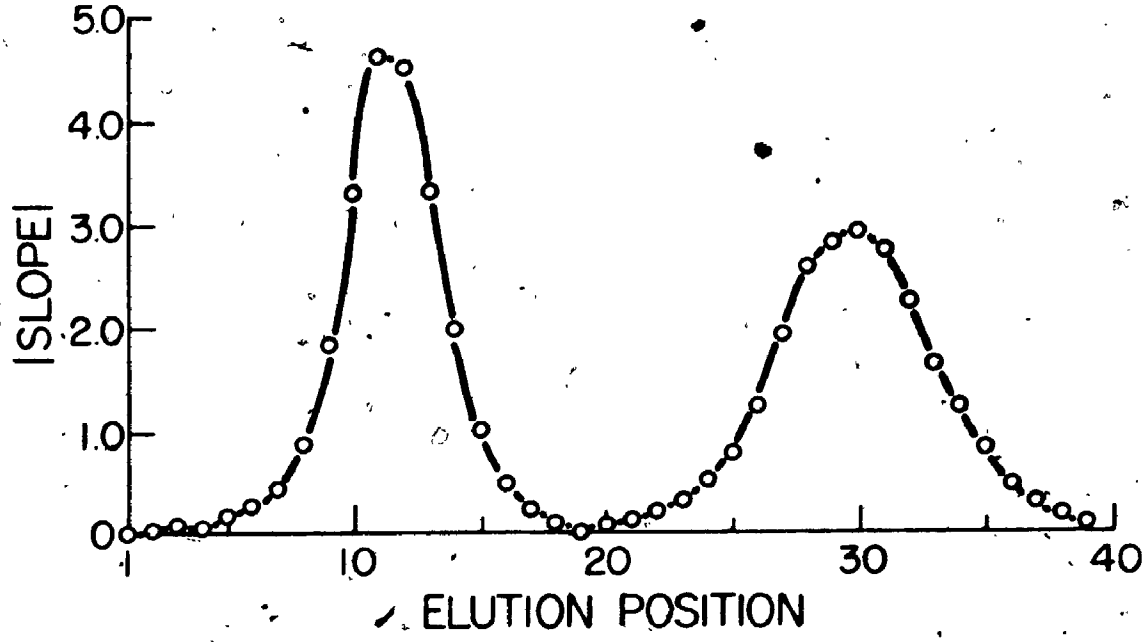


Figure 51: Plateau study slope graph of α^{SH} at two different concentrations. By monitoring the elution at 413 nm and 368 nm, a six-fold difference in subunit concentration was possible.

Upper: 413 nm; α^{SH} concentration = 6.5 uM

Lower: 368 nm; α^{SH} concentration = 31.4 uM


5.0
4.0
3.0
2.0
1.0
0
|SLOPE|
1
10
20
30
40
ELUTION POSITION


5.0
4.0
3.0
2.0
1.0
0
|SLOPE|
1
10
20
30
40
ELUTION POSITION

dialysis to pH 9.5 had the same effect. It appeared virtually immediately with the rise in pH and subsequently was not seen to increase during a twelve hour period. The amount present was estimated as less than two percent of the total protein and therefore not considered an obstacle to meaningful investigation of dimerization via crosslinking techniques, well suited for this purpose.

Crosslinking Studies

As discussed earlier, the hemoglobin α subunit is sufficiently stable, by all the investigative procedures employed, to incubation and reaction with imidoesters at pH 9.5 in PO_4 - EDTA or 0.1 M borate - 2×10^{-5} M EDTA. Crosslinking with the bifunctional dimethyl adipimidate (DMA) was therefore a valuable method of gaining further evidence of α subunit interaction. In all cases reaction conditions were those recommended by Lockhart and Smith (in press) for solely intramolecular DMA bridges. As a means of detecting any diversity of molecular weight in reagent-exposed samples and the required controls, the binding capacity apparatus involving a G-75 Sephadex column equilibrated in PO_4 - EDTA was used.

Reaction of α^{SH} with ethyl acetimidate resulted in no gel filtration elution asymmetry or mobility difference compared with that of untreated material. After concentration by ultrafiltration, the material was still seen to contain only monomer. Thus no imidoester-induced

association had occurred.

Reaction in a similar fashion with DMA produced inter-monomer crosslinks as proven by gel filtration and SDS-PAGE (Figures 52 and 53). Non-covalent interactions would certainly have been disrupted by these treatments. Two closely-spaced bands were seen in SDS-gels with mobility corresponding to that of dimer and apparently equivalent to those produced by the intramolecular DMA crosslinking of hemoglobin (Lockhart and Smith, in press). There was no evidence of trimer or tetramer.

Investigation by Sedimentation Equilibrium

The conventional sedimentation equilibrium technique in the analytical ultracentrifuge provides a direct method of estimating molecular weight and detecting association phenomena. If c is used to denote the protein concentration (as indicated by optical density recorded by the photoelectric scanner) and r the distance from the centre of rotation, then a graph of LOG c versus r^2 yields a slope which is proportional to apparent weight average molecular weight at every point in the cell. For a homogeneous, ideal solute, a constant slope results. If however, the plot curves upward, the solute is heterogeneous, has undergone degradation, or is self-associating.

Analysis of the concurrent equilibrium centrifugation of myoglobin, α^{PMB}, and α^{SH} resulted in the graphs

Figure 52: Sephadex chromatography of α^{SH} following reaction with dimethyl adipimidate. The method employed was that described earlier for the detection of hemoglobin binding capacity as a measure of functional haptoglobin concentration except that G-75 gel equilibrated in PO_4-EDTA was used.

Component identities assigned:

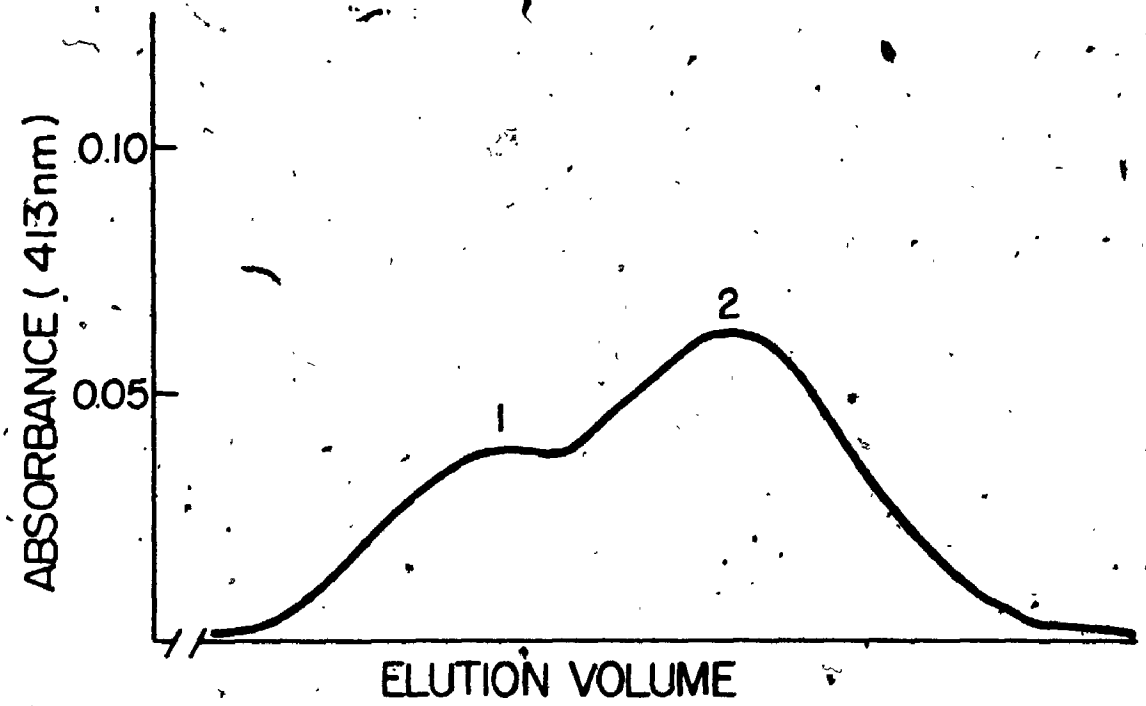
1. DMA-crosslinked dimer
2. DMA-modified monomer

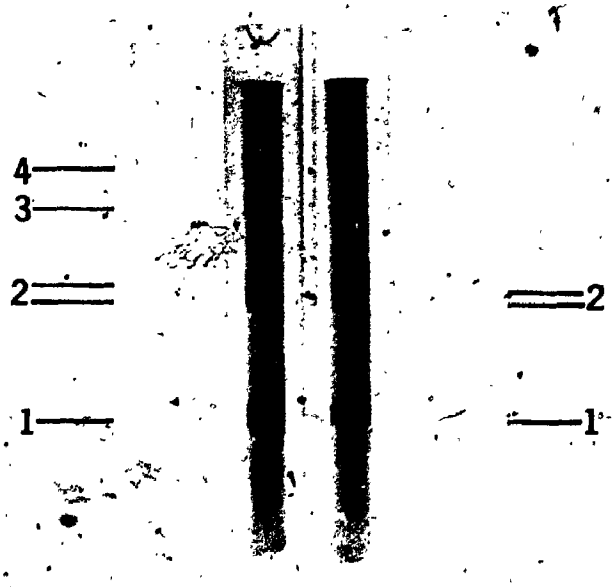
Figure 53: Analysis by SDS gel electrophoresis of α^{SH} following reaction with dimethyl adipimidate. Comparison is made with hemoglobin modified in the same way.

Left: DMA-reacted Hb

Right: DMA-reacted α^{SH}

Assigned band identities are: 1: monomer, 2: dimers, 3: trimer, 4: tetramer.


ABSORBANCE (413nm)
0.10
0.05
1
2
ELUTION VOLUME


4
3
2
1
2
1

presented in Figures 54, 55, 56. No departure from linearity is seen in that of myoglobin. Heterogeneity or degradation being highly unlikely, those of α^{PMB} and α^{SH} are evidence of subunit association. Table 8 compares the known molecular weights and those calculated from slopes at the meniscus and bottom of the cells. A value of 0.749 used for the partial specific volume ($\bar{v}$) of α subunit was that reported by Svedberg and Pedersen (1940) for hemoglobin. This agrees well with 0.748 calculated by Ranney et al (1965) for the subunit neglecting the contribution of the heme group. The $\bar{v}$ of myoglobin was taken as 0.743 (Ehrenberg, 1957). Construction lines in Figures 55 and 56 indicate the points used for slope estimates. The relative degree of association of α^{PMB} and α^{SH} is consistent with the plateau and crosslinking studies, with molecular weights approaching that of a dimer.

Investigation by Sedimentation Velocity

Dimer formation of α^{SH} and to a lesser extent α^{PMB} should also be reflected by increased sedimentation velocity relative to myoglobin, a non-associating protein of slightly larger molecular weight. $S_{20,w}$ values of concurrently sedimented myoglobin, α^{PMB}, and α^{SH} presented in Table 9 bear out this prediction.

Figure 54: Sedimentation equilibrium ultracentrifugation graph of LOG Absorbance versus x^2 for myoglobin. Absorbance is linearly related to protein concentration and x is the distance from the centre of rotation.
Prior to centrifugation, the myoglobin concentration was 11.7 u molar.

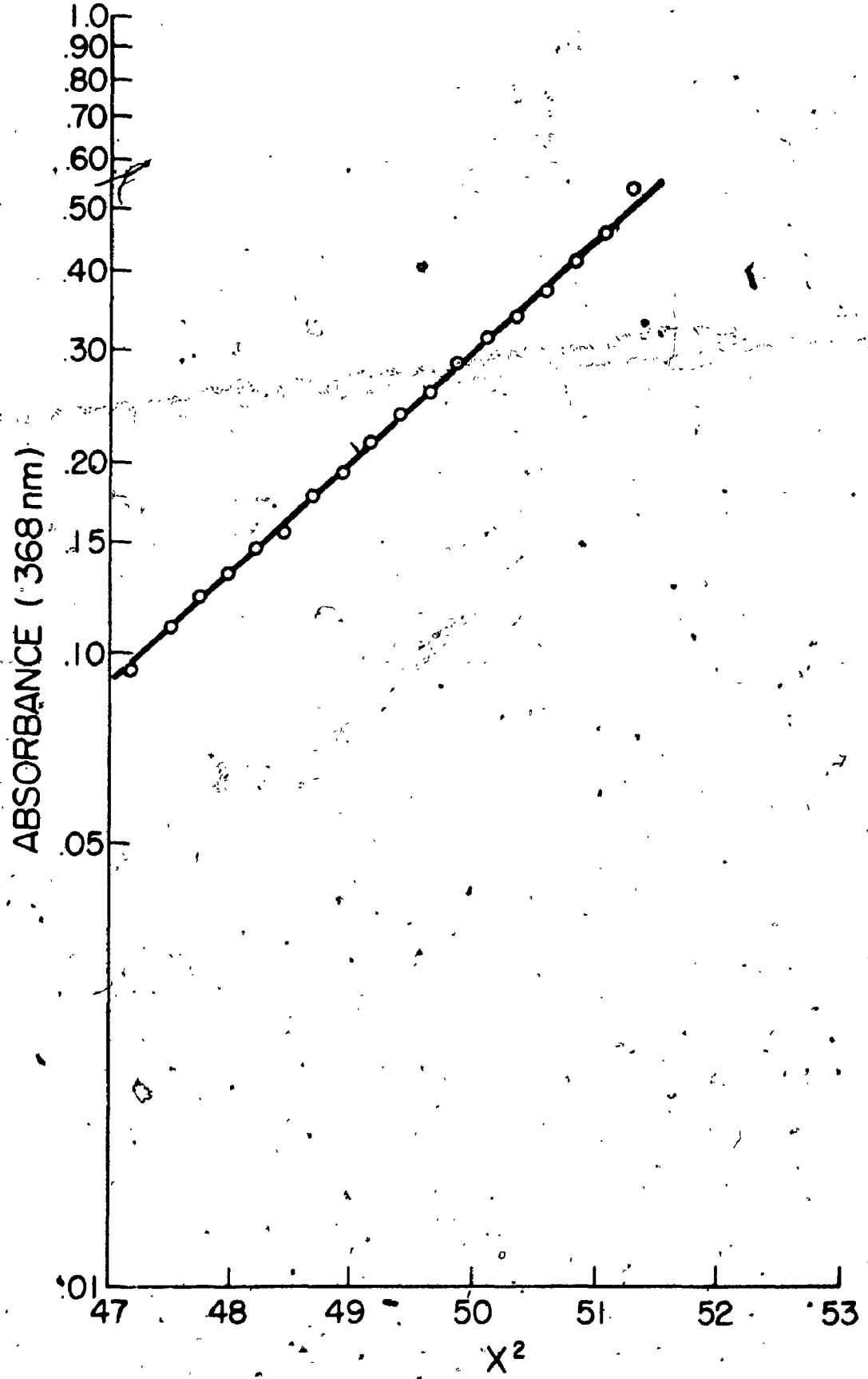

1.0
.90
.80
.70
.60
.50
.40
.30
.20
.15
.10
.05
.01
ABSORBANCE (368 nm)
47
48
49
50
51
52
53
X²

Figure 55: Sedimentation equilibrium ultracentrifugation graph of LOG Absorbance versus x^2 for α^{PMB}. Absorbance is linearly related to protein concentration and x is the distance to the centre of rotation.

Prior to centrifugation, the α^{PMB} concentration was 12.9 u molar.

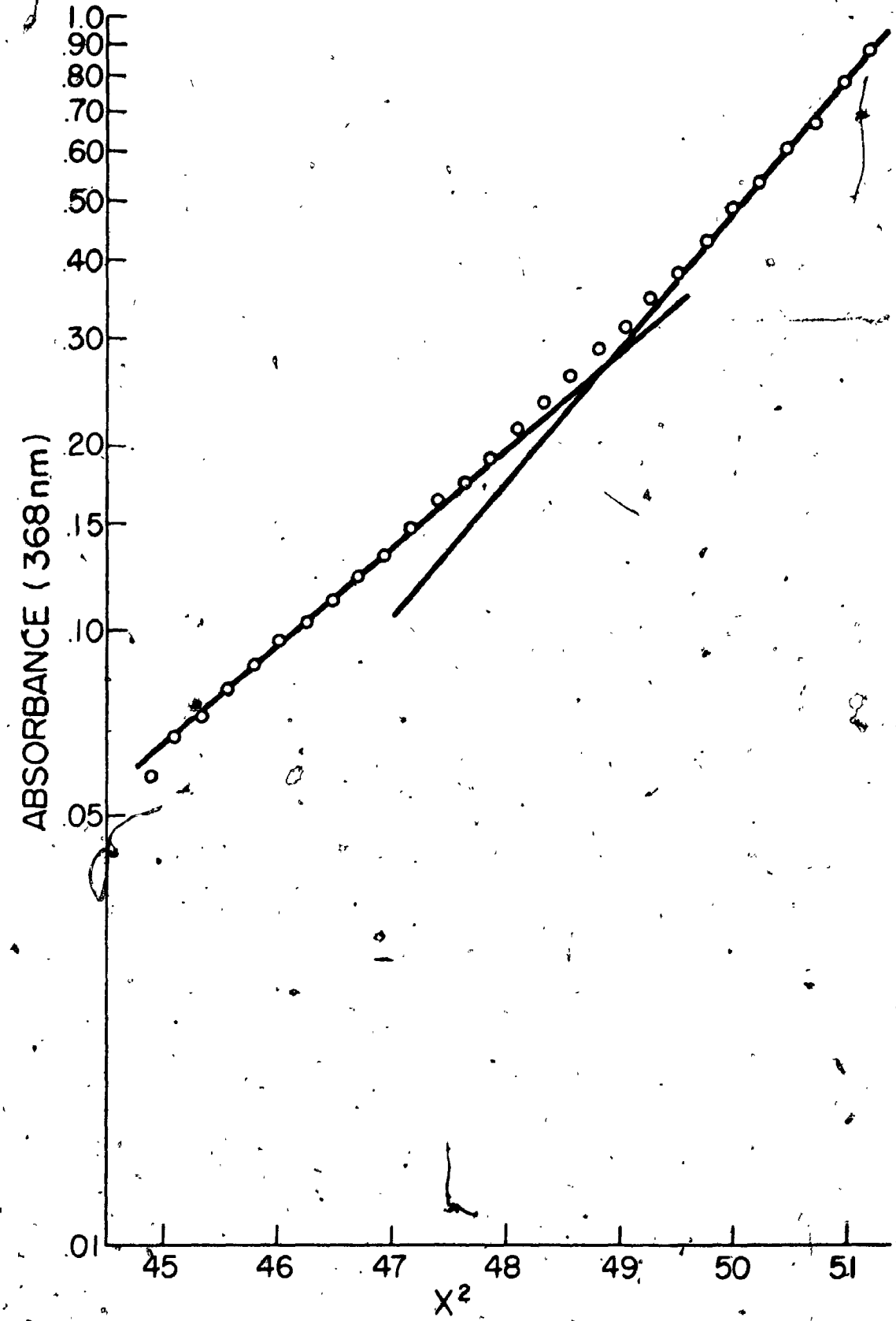
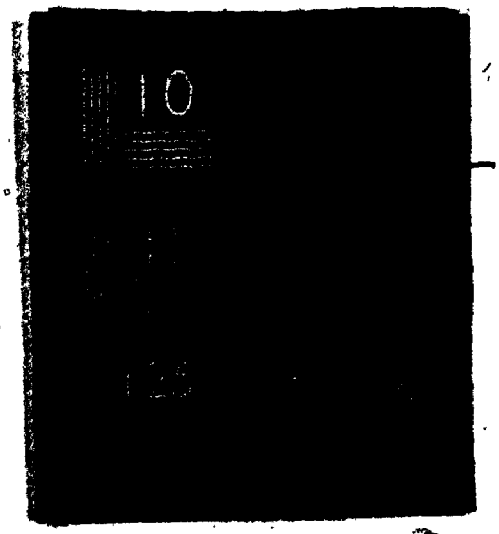

1.0
.90
.80
.70
.60
.50
.40
.30
.20
.15
.10
.05
.01
ABSORBANCE (368nm)
45
46
47
48
49
50
51
X²

Figure 56: Sedimentation equilibrium ultracentrifugation graph of LOG Absorbance versus x^2 for α^{SH}. Absorbance is linearly related to protein concentration and x is the distance to the centre of rotation.

Prior to centrifugation, the α^{SH} concentration was 12.1 u molar.

4 5

OF/DE



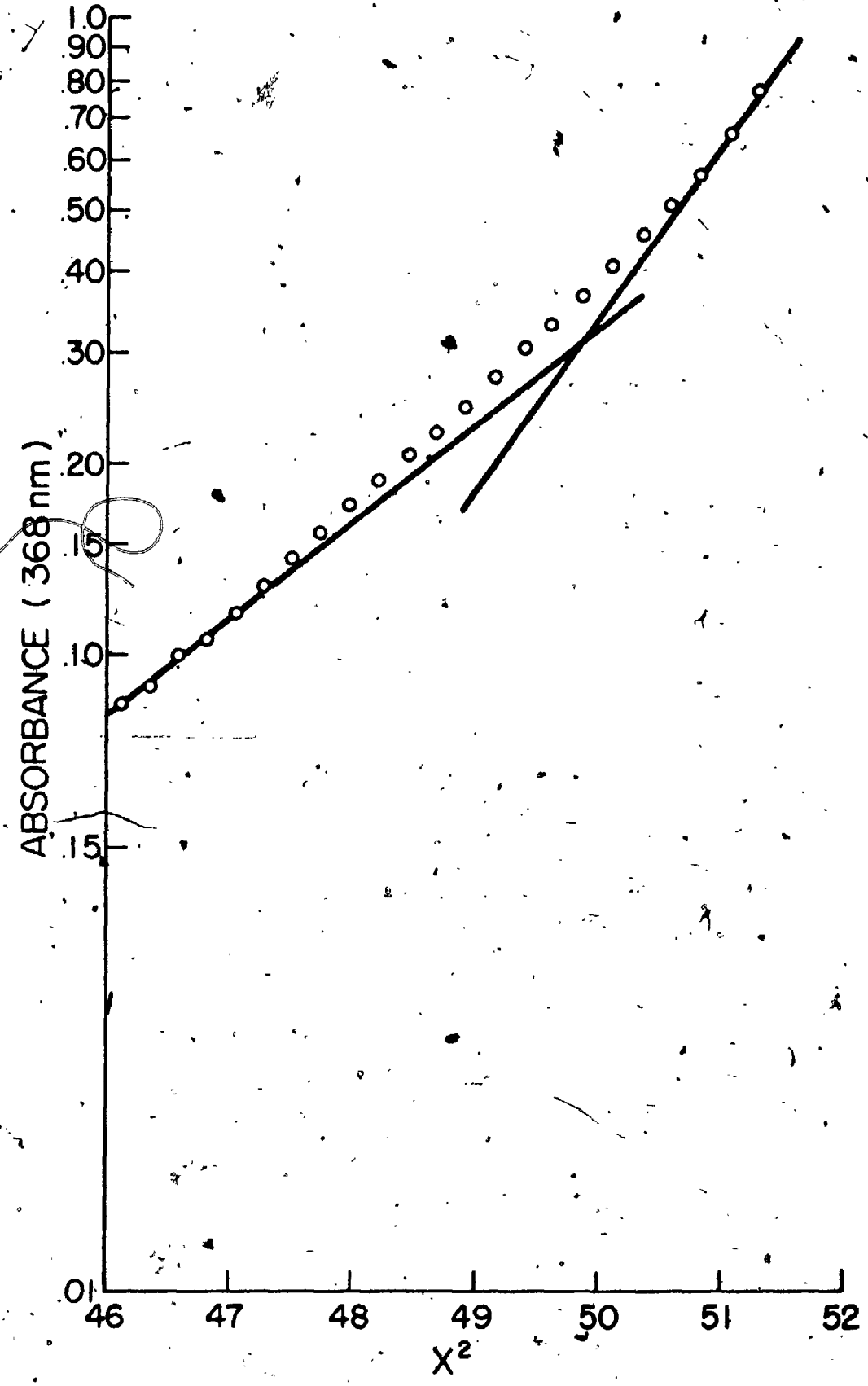

1.0
.90
.80
.70
.60
.50
.40
.30
.20
.15
.10
.15
.01
ABSORBANCE (368 nm)
46
47
48
49
50
51
52
X²

Table 8: Molecular weight of myoglobin, α^{PMB} and α^{SH} determined by sedimentation equilibrium ultracentrifugation. Since that of α^{PMB} and α^{SH} is apparently a function of concentration, values obtained at the cell meniscus and bottom are quoted. Comparison is made with literature values assuming a monomeric quaternary structure.

Table 9: Sedimentation coefficient of myoglobin, α^{PMB} and α^{SH}.

Protein	Molecular Weight as Monomer	Apparent MW * Meniscus	Cell Bottom	% Deviation Meniscus Value, Monomeric MW
Myoglobin	17,800	16,800		5.6
α^{PMB}	16,000	15,700	21,700	1.9
α^{SH}	15,700	14,900	26,800	3.8

* Molecular Weight

Protein	Concentration	Molecular Weight	$S^{-}_{20,w}$
Myoglobin	25 uM	17,800	1.84
α^{PMB}	28 uM	16,000	1.81
α^{SH}	29 uM	15,700	1.91

In all cases, symmetrical boundaries were observed. This is consistent with a system at equilibrium involving rapid association-dissociation (Gilbert, 1955; Nichol et al, 1964).

Kawahara, Kirshner and Tanford (1965) describe a method for determining the equilibrium constant of any system which involves reversible dissociation to half-molecules. Their application to the hemoglobin tetramer-dimer case can easily be extended to that of the α subunit dimer-monomer system. If therefore, dimer and monomer are designated A and B respectively, then the observed sedimentation coefficient is

$$\bar{S} = (1 - \gamma)S_A + \gamma S_B$$

where γ is the weight fraction of subunit in the dissociated form, and S_A and S_B are the sedimentation coefficients of A and B. Assuming no change in frictional ratio coefficient or preferential binding of solvent upon dissociation, S_B can be calculated as $(0.5)^{2/3}S_A = 1.8$ using their similarly calculated and experimentally verified value of $S_{dimer} = 2.90$

The S value here observed for α^{PMB}, which undergoes minimal dimerization, was 1.81 while that reported by Rosemayer and Huehns (1967) was 1.8. Thus with $\bar{S}$ = 1.91, S_A = 2.9, and S_B = 1.8, equation yielded γ = 0.91. From this, the dissociation constant was calculated as 5×10^{-4} moles/liter according to

$$K_d = \frac{4\gamma^2 c_o}{(1-\gamma) M}$$

where c_o is the protein concentration in g/liter and M is the molecular weight of dimer. The free energy of dissociation is therefore

$$\Delta G_d^o = -RT \ln K_d$$
$$= 6.0 \text{ k cal/mole}$$

Confidence in the accuracy of these constants should be tempered by noting that the value of γ involved in their calculation was at the upper limit of the $0.1 < \gamma < 0.9$ range recommended by Kawahara et al (1965). Repeating the study, with more extensive dimerization favoured by higher subunit concentration, would result in a value of γ nearer 0.5 and hence negligible error in K_d and ΔG_d^o. Schlieren optics, useful in protein concentrations of 1 to 10 mg/ml, would be required as the maximum attainable for hemglobin with the photoelectric scanner at 368 mu is 0.6 mg/ml.

Effect of Ionic Strength of Subunit Association

Many authors including Antonini et al (1962) and Kawahara et al (1965) have reported and discussed the effect

of various salts and other small molecules on hemoglobin dissociation as a valuable probe of the non-covalent forces which bind the subunits. The effect of high ionic strength on α subunit association was therefore investigated.

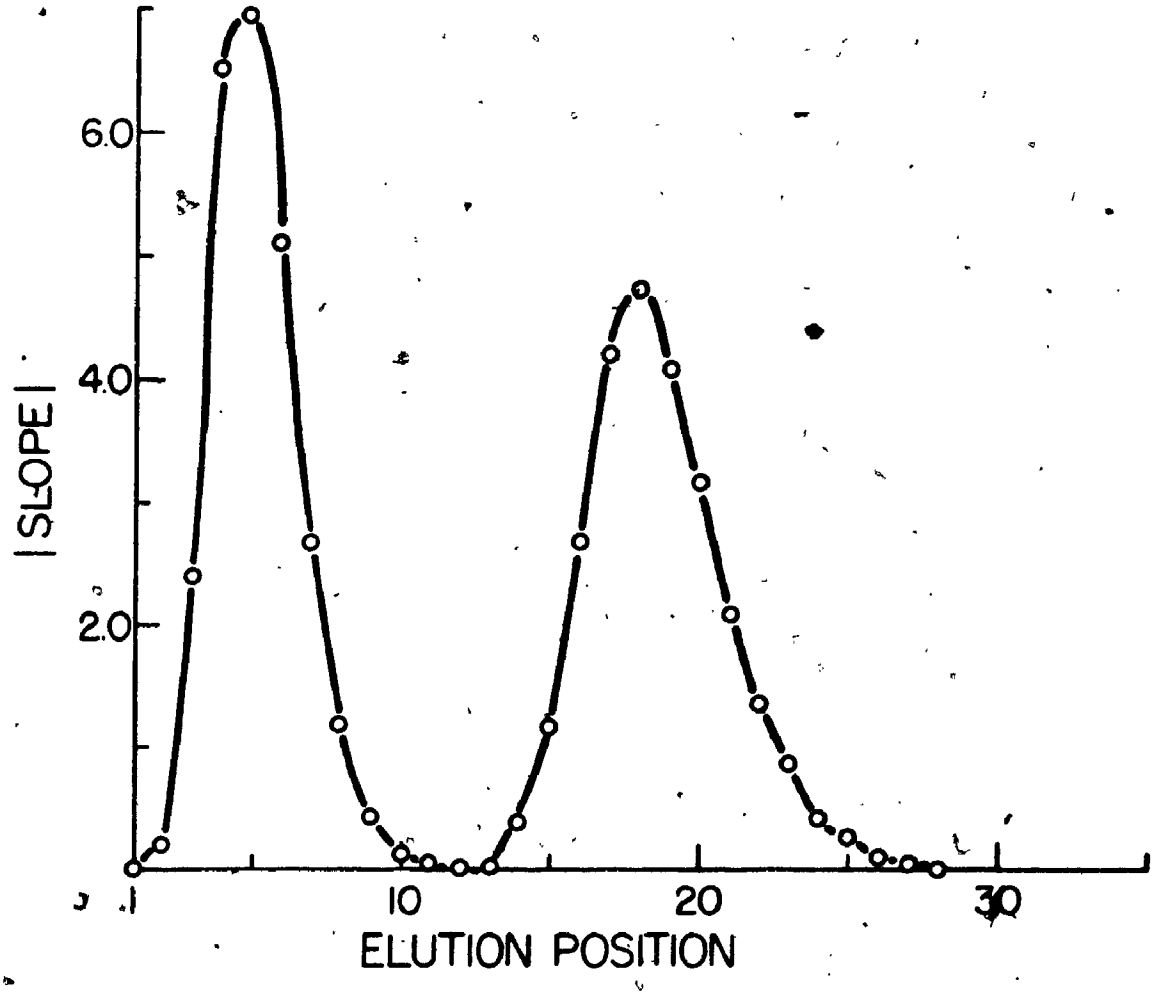
Gel Filtration Plateau Studies

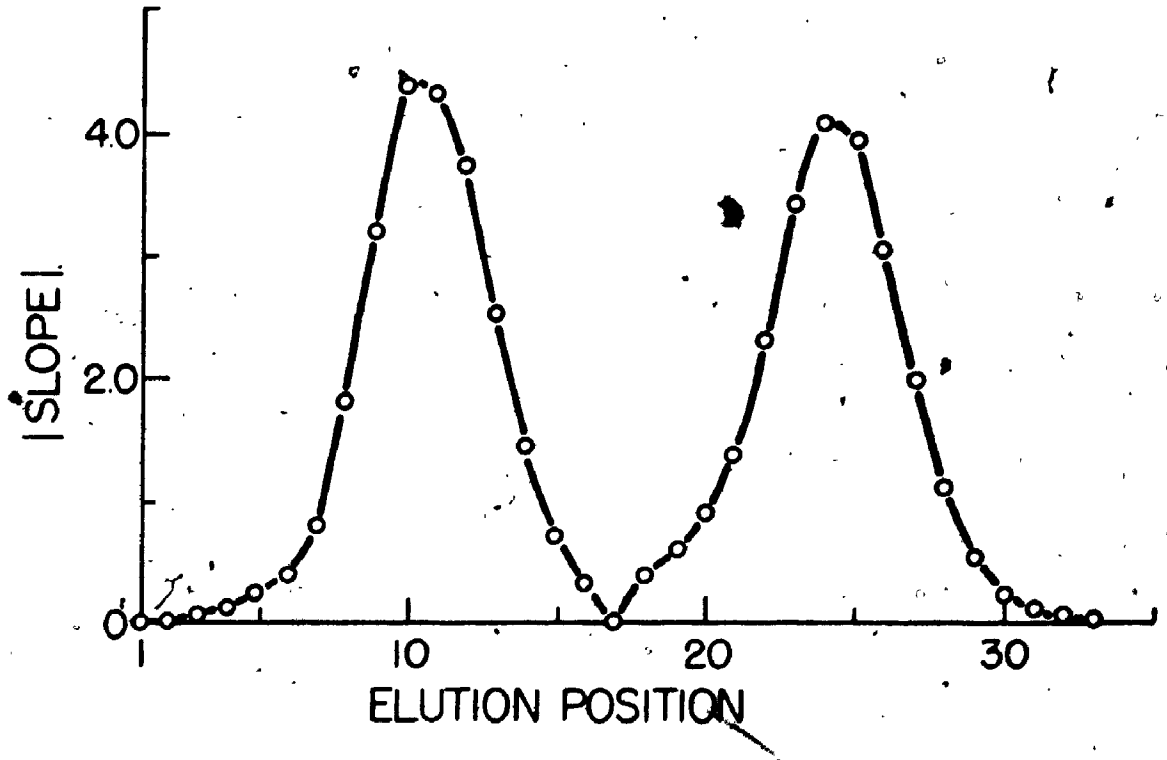
In view of the promotion by high molar salt of virtually complete dissociation of hemoglobin tetramer to dimers, plateau studies of α^{SH} in 2 M NaCl revealed an initially surprising phenomenon. Rather than abolition of association, there was extensive promotion. Myoglobin showed no signs of association. Conclusions from similar treatment of α^{PMB} were indeterminant as the profile was complicated by non-reversible aggregation similar to that seen in the study at pH 9.5. Appropriate profiles are presented in Figure 57.

Crosslinking Studies

Crosslinking α^{SH} subunits in 2 M NaCl with DMA produced a somewhat increased amount of dimer compared to the no-salt case as seen by G-75 gel filtration but much of the material was still monomeric. However crosslinking is not a direct assay of dimerization as the cross-link sites may be less favourable than those producing intra-subunit bridges. Thus no inconsistency exists with the extensive promotion of association detected by gel

Figure 57: Plateau study slope graph of α^{SH} (upper) and of α^{PMB} (lower) in 2 molar NaCl.


6.0
4.0
2.0
|SLOPE|
1
10
20
30
ELUTION POSITION


4.0
2.0
0
|SLOPE|
1
10
20
30
ELUTION POSITION

filtration.

SDS gel electrophoresis of α^{SH} crosslinked in the presence and absence of 2 M NaCl followed by gel band densitometry indicated an increase of the total protein present as dimer from 42 to 60 per cent (Figure 58). No estimate of the relative amounts of dimer components was possible due to densitometry-exaggerated overlap, but the one of greater mobility was seen to have increased in the presence of salt.

Both these crosslink preparations contained three bands in PAGE at pH 8.9 (Figure 59). A confident assignment of identity could not be made but the slowest and fastest could be the two dimers observed in SDS-PAGE. The band of intermediate mobility would therefore be monomer. This separation suggests preparative gel electrophoresis of the material and determination of the number of subunits in each component. From this point, investigation of individual ability to bind haptoglobin would be an interesting route towards a topographical characterization.

Sedimentation Velocity Studies

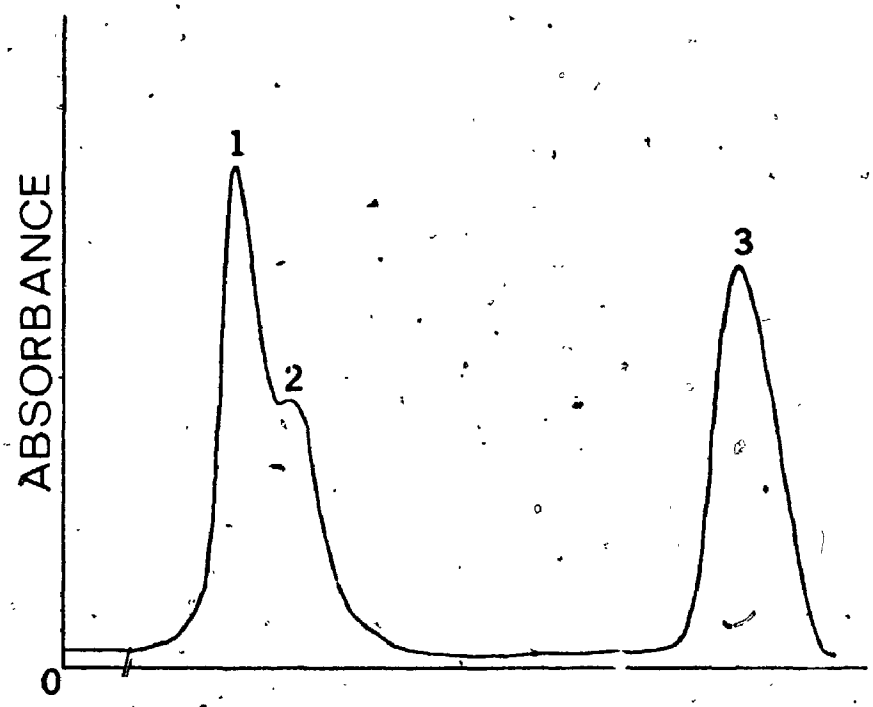
Sedimentation velocity analysis contributed further to the topic of ionic strength and α^{SH} subunit association. The $S_{20,w}$ value of α^{SH} in the presence of 2 M NaCl was seen to have risen to 2.30 from 1.91 in PO_4 - EDTA while that of Hb fell from 4.06 to 2.81 in the same conditions.

Figure 58: Densitometry patterns of α^{SH} crosslinked with DMA in the absence (Profile A) and presence (Profile B) of 2 molar NaCl following separation by SDS gel electrophoresis.

The graphical zero refers to the top of the gel.

Assigned identities:

1. Lower-mobility dimer or "slow dimer"
2. Greater-mobility dimer or "fast dimer"
3. Monomer


ABSORBANCE
1
2
3
0

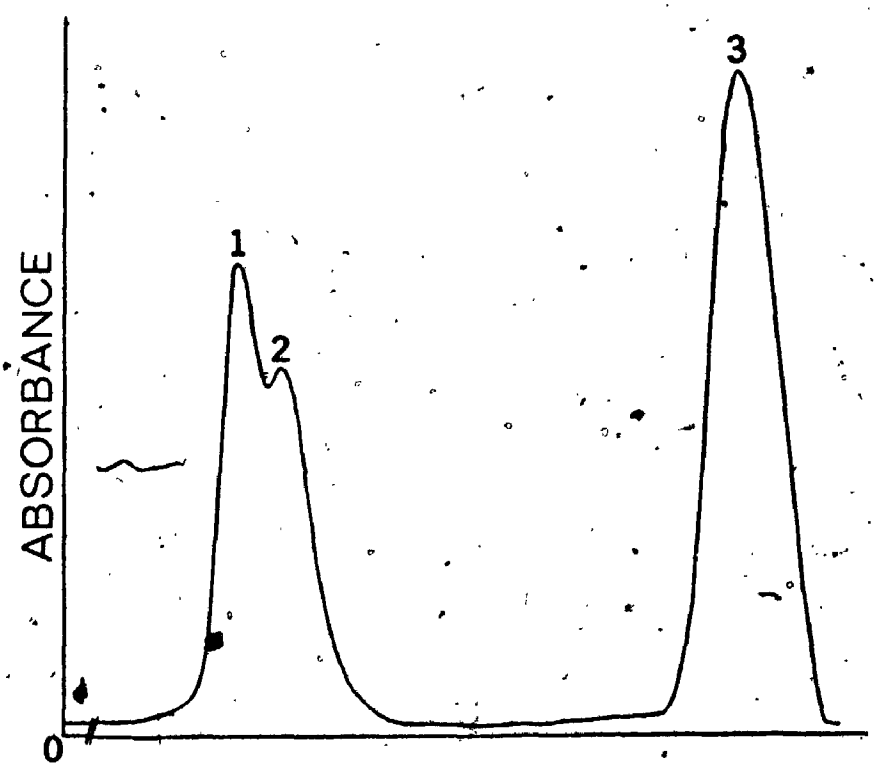
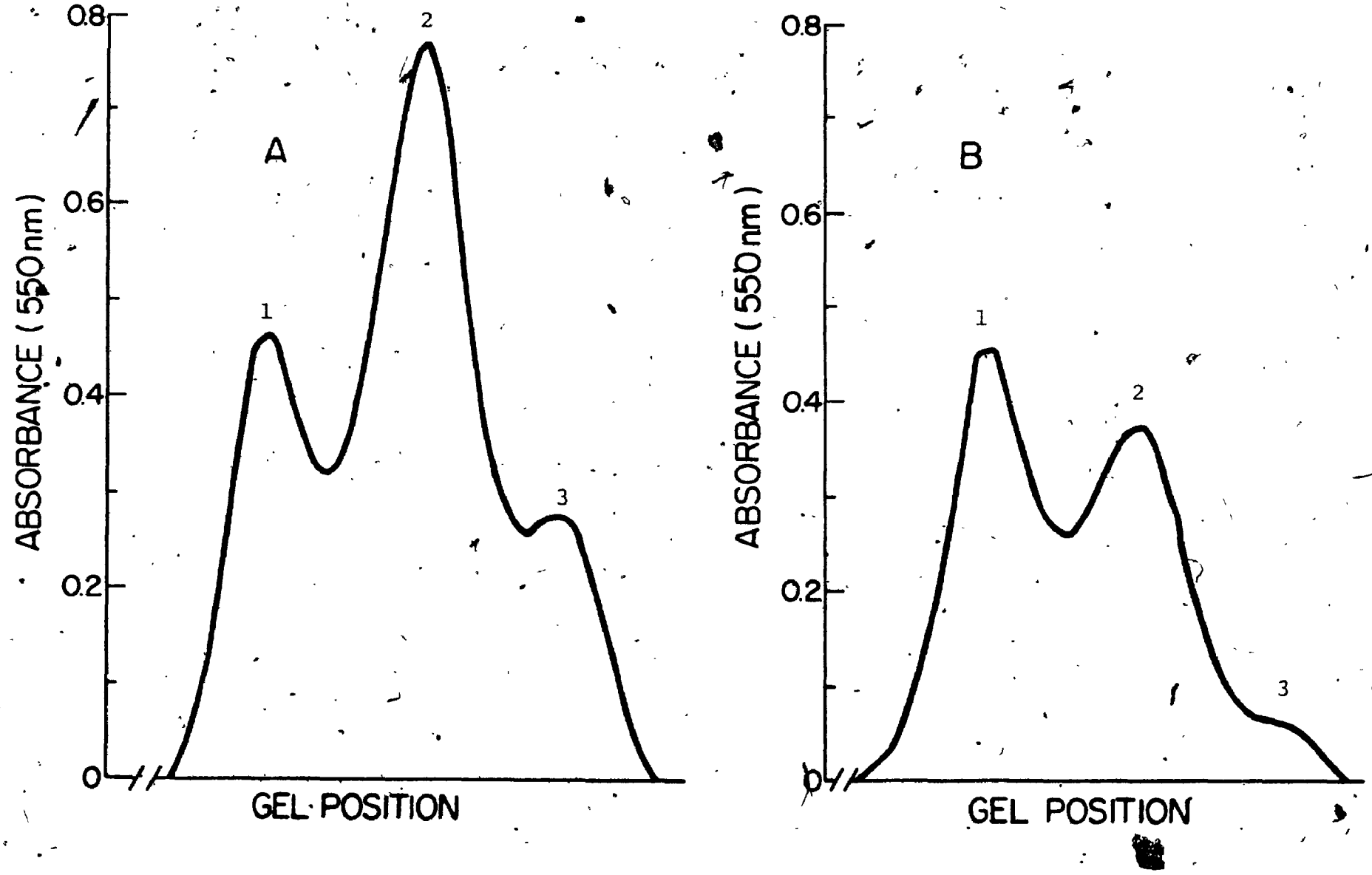

ABSORBANCE
1
2
3
0

Figure 59: Densitometry patterns of α^{SH} crosslinked with DMA in the absence (Profile A) and presence (Profile B) of 2 molar NaCl following separation by analytical gel electrophoresis. The graphical zero refers to the top of the gel.

Tentative band identities are:

1. Crosslinked dimer
2. Monomer
3. Crosslinked dimer


A
B
ABSORBANCE (550nm)
0.8
0.6
0.4
0.2
0
1
2
3
GEL POSITION

That of hemoglobin in the absence of sodium chloride compares well with 4.13 reported by Chiancone, Vecchini et al (1966) in similar conditions. These data are presented in Table 11.

Utilizing the method of Kawahara et al (1965) described earlier, the dissociation constant of dimer α^{SH} in 2 M NaCl-PO_4-EDTA was calculated as 4.2×10^{-5} M and the standard free energy of dissociation as 7.9 kcal/mole. Considerable accuracy is involved as the value of γ, the weight fraction of subunit as monomer, was 0.545, that is, near the centre of its range. The ΔG_d^o at the weaker ($\alpha_1\beta_2$) contact surface of hemoglobin is 8.2 kcal/mole in 0.02 M salt at neutral pH (Kawahara et al, 1965). Thus the effect of high salt concentration near pH neutrality on isolated α^{SH} subunits is to make the environment sufficiently unattractive such that the tendency to associate as a dimer is similar to that of $\alpha\beta$ dimers to form hemoglobin in moderate concentration of salt.

Evidently the need to escape the high ionic strength is characteristic of only part of the α subunit surface and is satisfied by dimeric association. If this were not the case, higher order irreversible aggregation leading to denaturation would be expected. This is known not to occur as the subunits remain native within the time scale of sample preparation and use in the plateau study technique which involves of the order of four hours at 22°C.

Table 10: Effect of 2 molar NaCl on the sedimentation velocity of α^{SH} and hemoglobin.

Table 11: Effect of brief exposure to 3 molar NaCl on the sedimentation coefficient of α^{SH}.

Protein	[NaCl] (M)	$S_{20,w}$
α^{SH}	0.0	1.91
	2.0	2.30
Hemoglobin	0.0	4.06
	2.0	2.81

$[NaCl]_{Final}$	Brief Exposure to 3M NaCl	$S_{20,w}$
0.5 M	Negative	1.90
0.5 M	Affirmative	1.97

The free energy of dissociation of Hb to dimers is a function of pH (Field and O'Brien, 1955; Hasserodt and Vinograd, 1959) but not of temperature (Hasserodt and Vinograd, 1959; Kirshner and Tanford, 1964). Regretfully, determination of the effect of these parameters in the case of α dimer dissociation lies in the future.

α^{SH} Native State in Sodium Chloride

In their study of hemoglobin quaternary dissociation to dimers, Kirshner and Tanford (1964) observed apparent denaturation induced by sodium chloride which began at a molarity above three. No samples in the present work involved incubation in that concentration range. The maximum molarity employed was 2.0. However subunits were mixed with 3 M NaCl to increase ionic strnegth with a minimum of protein dilution. For this reason it was necessary to investigate the effect, if any, of this exposure.

Two samples of α^{SH} were treated such that they had identical final ionic conditions of 0.5 M NaCl in PO_4 - EDTA but different histories. The first was diluted with two volumes of 0.75 M NaCl-PO_4-EDTA. The second was added to one half volume of 3 M NaCl-PO_4-EDTA and the resulting solution was diluted 1:1 with PO_4 - EDTA. Upon ultracentrifugation, the sedimentation coefficients of these two samples were 1.90 and 1.97 respectively (Table 10). Thus brief exposure to 3 M NaCl in the preparation of samples of lower sodium chloride concentration had no adverse effect

on the subunit native state. This conclusion is reinforced by the plateau studies of α^{SH} in 2 M NaCl-PO_4-EDTA described above. Very slight salt-induced denaturation could have been detected by a leading shoulder on the elution profile and by a sloping plateau region. Neither was observed.

Discussion of α Subunit Association

By several methods, the reversible association of α subunits to form dimers has been observed.

The degree of association was consistently greater in the case of α^{SH} as compared with that of α^{PMB}. This effect of PCMB modification of the 104 sulfhydryl on the association of the ferrous subunit is in contrast to that of the ferric derivatives. Bucci and Fronticelli (1971) reported that ferric α^{PMB} subunits in 0.5 M acetate buffer (pH 5.2) exist as a reversibly associating system with dimers the predominant species. Ferric α^{SH} subunits aggregate to a lesser extent and sediment essentially as monomers. However heme iron oxidation is accompanied by definite gross conformational rearrangement as evidenced by changes in the circular dichroism spectrum, complement fixation curve, and the ability to interact with ferrous subunits. These authors have suggested that the migration of the heme pocket distal histidine to form a hemichromagen is the initiating event. Thus it is not suprising that the ferrous α^{SH} and α^{PMB} association behavior does not parallel that of the ferric forms.

Although no direct evidence has been obtained as to the surfaces involved in the dimerization of α subunits, something of its nature has been learned. Drawing on the extensive knowledge of hemoglobin structure in the literature, certain insight can be gained.

Elevated concentrations of neutral electrolytes such as sodium chloride are known to weaken polar interactions and to strengthen non-polar ones. Thus the increased dimerization of α subunits by 2 M NaCl can be attributed to the involvement of non-polar surfaces. Hemoglobin dissociation to $\alpha_1\beta_1$ dimers is enhanced by high ionic strength which implies a very significant contribution of polar interactions to the $\alpha_1\beta_2$ contact. Perutz (1968) has indicated that although only two H-bonds are detectable in this region, their strength may be unusually high due to their existence in an area where water is largely excluded. Further dissociation to monomers is not promoted by a host of salts which are completely effective in causing formation of dimers by Hb (Kirshner and Tanford, 1964). This illustrates a basic difference between the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ interfaces. Figure 4 presents the interactions involved in each as defined by inter-atomic distances of 4 Å or less from data obtained X-ray crystallographically with horse oxyhemoglobin (Perutz, 1968). (The structural pattern of α and β subunits and their integration into a tetramer seems to be a fundamental design which is common across much of the evolutionary scale. This makes struc-

tural information of mammalian hemoglobins, specifically horse and human, directly comparable.)

Bonding in both these contact areas is seen to be highly non-polar. With GLY, ALA, VAL, LEU, PHE, CYS, MET, TRP, and TYR considered participants, there are eight and five hydrophobic interactions respectively in the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ regions. If TYR is excluded from this set of amino acids, being a marginal member due to its hydroxyl function, the numbers become eight and three. Kawahara et al (1965) describe the $\alpha_1\beta_2$ region as being not primarily non-polar. The $\alpha_1\beta_1$ region is therefore the more extensive and reluctant to be exposed to an aqueous environment. This is further illustrated by the lack of detectable dissociation of Hb to monomer at concentrations as low as 10^{-8} molar (Bucci, 1971; Kellet and Schachman, 1971). The contact surfaces between like subunits are polar in nature and few in number. The same is true for subunit areas which are exterior in the tetramer. Even in the crystal form, the contacts between neighbouring Hb molecules involve no more than a few polar atoms. This fact, coupled with the observed salt effect, eliminate them as possible candidates for dimerization. Thus the surface strongly implicated is that which joins with β to form the $\alpha_1\beta_1$ dimer.

The $\alpha_1\beta_1$ interface is an extensive area of complementary and interlocking structure (Perutz, 1969). This is unlikely in α_2 despite the considerable tertiary

similarity between the α and β subunits and must result in an imperfect fit and hence instability. Another probably contributing factor is the loss of suitable H-bonding of two polar residues, ASP 126 and HIS 103. In addition, the carboxylate group of the former would be expected to strongly inhibit a tight interaction in an α dimer due to ion repulsion. Both ASP126 and HIS103 are located at an interior edge of the $\alpha_1\beta_1$ dimer when included in a tetramer, and form part of the internal cavity. As part of a free dimer, they would be well exposed to the medium. Thus, assuming a similar orientation of two α forming a dimer, salt would decrease the electrical potential fields created by these polar centres and allow closer approach for greater meshing of hydrophobic groups.

Alpha subunits CYS 104 is known to form part of the $\alpha_1\beta_1$ contact region in horse hemoglobin (Figure 4) and in human hemoglobin to "lie in the interior cavity tucked away between [two] helices ...On dissociation of the molecule into αβ-dimers, steric hindrance would still prevent their reacting with mercurials carrying large polar groups, such as paramercuribenzoate" (Perutz, 1965). At neutral pH, reaction does not occur without prior destabilization of the dimer by PMB introduction at β 112. This is implied by studies involving hemoglobins lacking a β 112 sulfhydryl in which dissociation proceeds only as far as dimer after modification at β 93. α 104 is

unavailable for reaction. In normal hemoglobin, recombination of α with the β subunit subsequent to CYS 104 modification is unfavourable (Rosemeyer and Huehns, 1967). A similar effect would be predicated if this surface of α was involved in the α dimer. This is, in fact, observed experimentally.

The proposed mode of association being true, then the α subunit surface which normally comprises part of the $\alpha_1\beta_2$ contact must still be exposed in α_2. Elements which would conceivably discourage the addition of a third α are the more polar nature of the $\alpha_1\beta_2$ contact, poor complementarity, and possible repulsion from the ASP and TYR residues mentioned above. Association of two α dimers as a tetramer would also seem unlikely. High ionic strength should make this structure even less favoured. As determined by SDS-PAGE, DMA crosslink products of α^{SH} with and without the presence of 2 M NaCl produce no trimer or tetramer. This is also evidence against denaturation and salting out.

Different positions of crosslink in a single dimer would be expected to result in variation of shape and mobility in SDS gels. This would explain the two observed dimer components in a way consistent with the above proposal. (This is not to say that each of these dimer bands do not consist of several subfractions.) An unequivocal resolution of this point can only come with peptide

analysis. For the present, the above discussion speaks in favour of association of α subunits to form dimers analogous to that preferred by α and β subunits.

DMA α^{SH} Species and Their Interaction with Hp

The interaction of crosslinked α subunits with haptoglobin was investigated by means of passage through a G-75 column equilibrated in PO_4 - EDTA as part of the binding capacity system. Only complex involving strong interactive forces sufficient to make its formation virtually irreversible (as in the case of HpHb) would survive this chromatographic journey. The detection of weaker interaction, requiring methods applied to the study of unmodified α subunit binding to Hp, was not attempted.

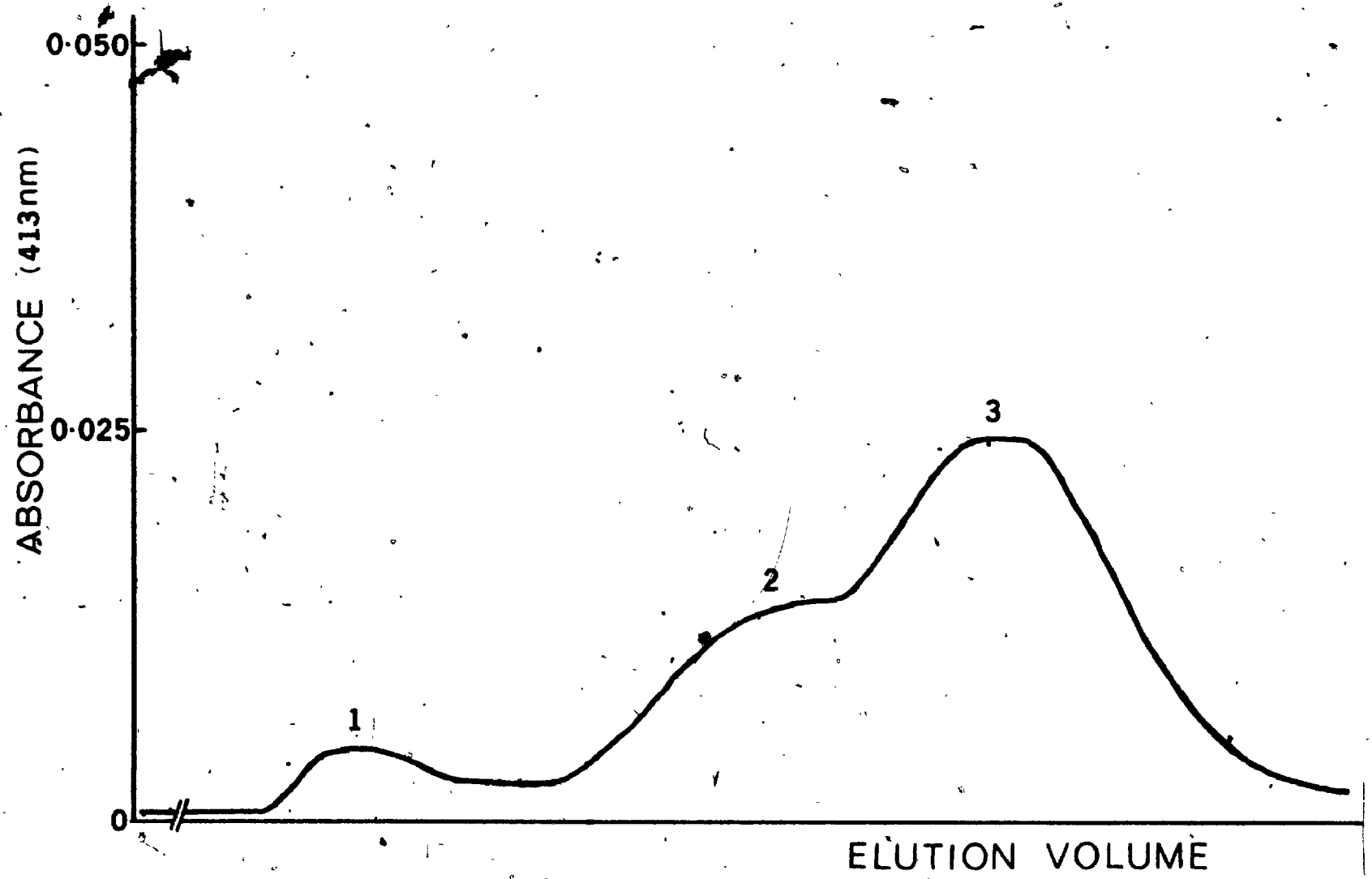
No sign of stable complex was observed upon the Sephadex separation of haptoglobin added to ethyl acetimidate-reacted α^{SH}, but the presence of strong interaction between Hp and DMA cross-linked subunits was definite (Figure 60).

Reaction of α^{SH} with the bifunctional imidoester to form solely intramolecular bridges necessitated low protein concentration. The addition of Hp subsequent to mopping up unreacted DMA with glycine involved further dilution. Component separation through Sephadex G-75 reduced the concentration still further. Thus the peak intensities in the elution profile were of the order of 0.1 optical density units. This made it difficult to

Figure 60: Sephadex chromatography of haptoglobin added to DMA-reacted α^{SH}. The method used was that described earlier for the detection of Hb binding capacity as a measure of functional haptoglobin concentration except that G-75 gel was substituted for G-100 and the column was equilibrated in PO_4-EDTA.

Component identities assigned:

1. Haptoglobin DMA-crosslinked α^{SH} complex
2. Free DMA-crosslinked α^{SH} dimer
3. Free DMA-reacted α^{SH} monomer


0·050
0·025
0
ABSORBANCE (413nm)
1
2
3
ELUTION VOLUME

determine, upon comparison with a no-Hp blank, which of the two DMA-subunit components were binding to Hp or if indeed both were capable of forming stable complex. Concentration of DMA- α^{SH} was clearly required.

Micro-cell ultrafiltration was useful for this purpose. A qualitative estimate of the amount of subunit adsorption to the Diaflow membrane indicated that considerably less material had been lost in this way compared to that seen earlier with unmodified α^{SH}. It is conceivable that DMA crosslinks complement the secondary and tertiary forces in resisting loss of native conformation. In addition, if dimer formation reduces exposure of α^{SH} surface area(s) which are prone to membrane adhesion, covalent prevention of dissociation to monomers would decrease this source of interaction. The slight amount of particulate matter present in the ultrafiltered DMA-α^{SH} was removed by Millipore filtration.

Separation of monomer and dimer at 5° C was carried out with the 2.5 x 90 cm G-75 column also employed for final purification of α subunits (Figure 61). A third and very minor component, of higher molecular weight, appeared as a leading shoulder of the dimer peak. This presumably consisted of aggregated material of insufficient size to be removed by a 0.45 μ Millipore filter.

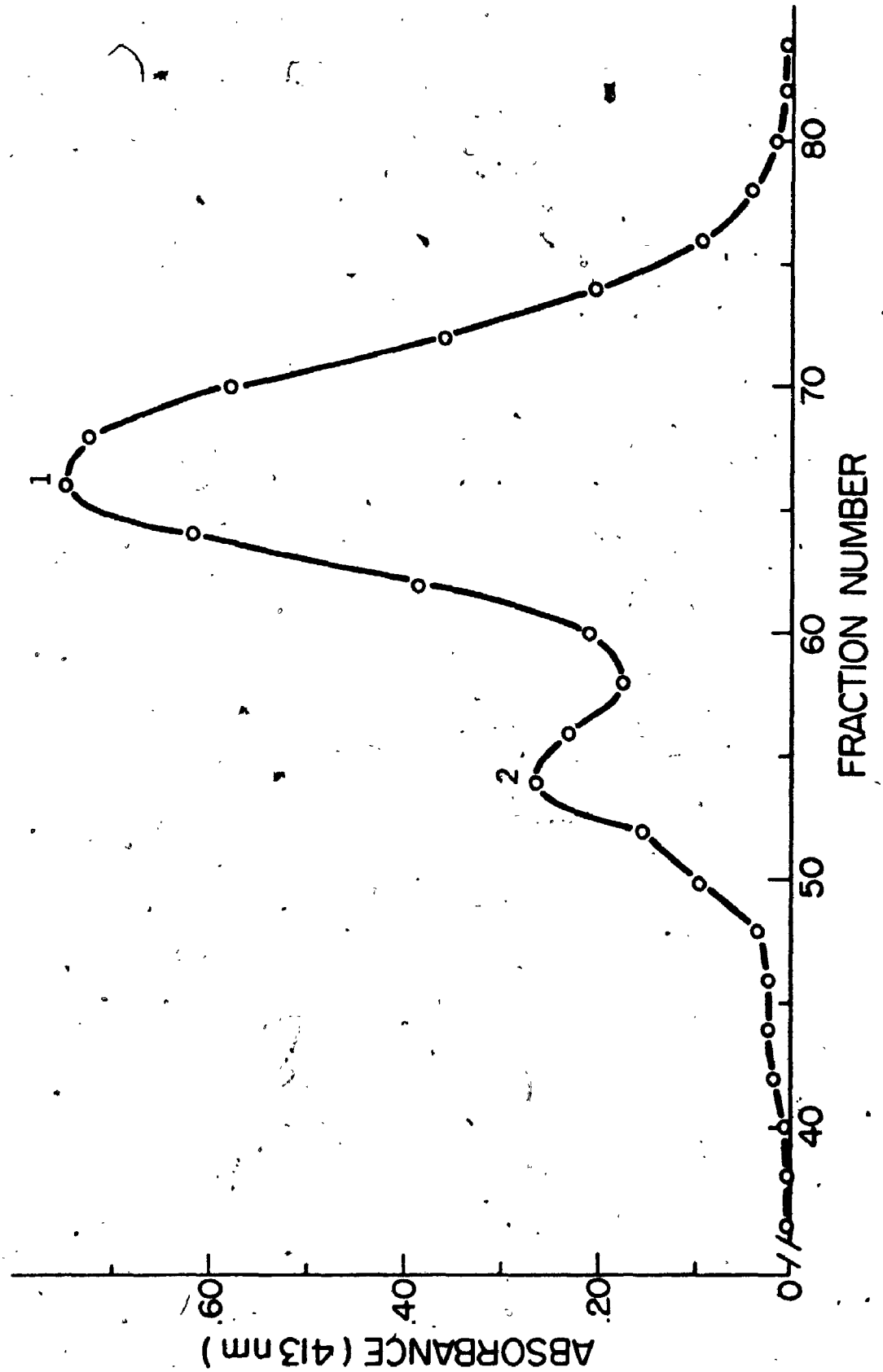
An assay of ability to bind strongly to haptoglobin

Figure 61: Preparative scale Sephadex chromatography of α^{SH} following reaction with dimethyl adipimidate. A 2.5 X 90 cm G-75 column equilibrated with PO_4-EDTA was employed. Prior to chromatography, the sample was concentrated by ultrafiltration and then Millipore filtered (0.45 μ pore size).

Component identities assigned:

1. DMA-reacted α^{SH} monomer
2. DMA-crosslinked α^{SH} dimer

The two components were later assayed for their ability to bind Hp. (Aliquots from the peak tubes were used). The dimer was reassayed following concentration by ultrafiltration of the fractions indicated.


ABSORBANCE (413 nm)
0
20
40
60
40
50
60
70
80
FRACTION NUMBER
1
2

was positive for the dimer and negative for the monomer (peak tubes used). In the former case, concentration of complex was extremely low. Use of that dimer material as indicated in the profile after ultrafiltration was more convincing (Figure 62). A similar assay with ultrafiltered and millipored ethyl acetimidate-α^{SH} revealed no stable complex.

It is apparent that all of the dimer DMA-α^{SH} does not bind equally well to Hp. Lockhart and Smith (in press) have noted that intramolecularly crosslinked DMA-Hb exhibits considerably reduced ability to complex and that fraction which can bind when examined by SDS-PAGE consists of the faster moving of two dimers and material dissociable to monomers. Fractionation of dimer-α^{SH} on the basis of interaction with Hp was attempted but insufficient protein was isolated for analysis.

If the α subunit associates solely as a dimer analogous to $\alpha_1\beta_1$, then all of the crosslinked material must be of this form. Thus the two-fold variation of mobility in SDS-PAGE and competence to bind Hp would be due to steric hindrance and/or conformational constraints introduced by DMA bridges, not to different modes of association. This question would best be resolved by peptide mapping.

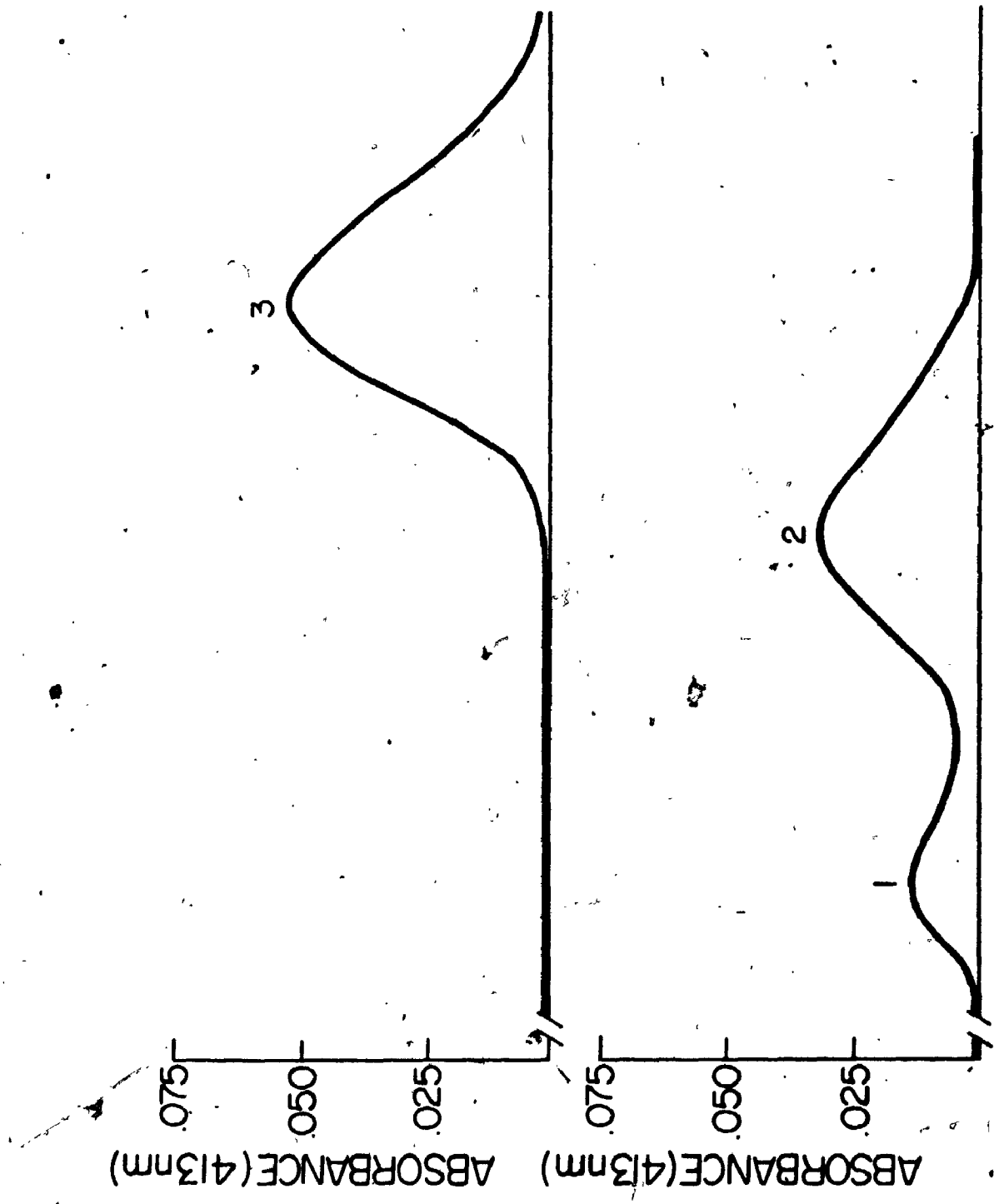
Crosslinking α^{SH} with DMA in the presence of Hp resulted in a greatly increased amount of complex and a

Figure 62: Assay of the ability to bind haptoglobin of DMA-reacted α^{SH} monomer (upper profile) and of DMA-crosslinked α^{SH} dimer (lower profile). Both of these α preparations were obtained from the elution presented in Figure 61. The DMA-α^{SH} dimer material was ultrafiltered prior to this assay.

Assigned component positions:

1. Hp DMA-crosslinked α^{SH} dimer complex
2. DMA-crosslinked α^{SH} dimer
3. DMA-reacted α^{SH} monomer

In both samples, the α^{SH} concentration (as monomer) was approximately 5 uM and that of Hp was 2 uM.


ABSORBANCE (413nm)
.075
.050
.025
1
2
ABSORBANCE (413nm)
.075
.050
.025
3

reduced amount of dimer compared with that seen in the case of Hp added to DMA-α^{SH} (Figure 63). This indicates that a very large proportion of the dimeric α^{SH} is capable of interaction with haptoglobin and becomes covalently linked to it when the complex is treated with the bifunctional imidoester. Also it implies that dimer dissociation from the Hp surface, before covalent attachment is established, is short lived. If this were not the case, the amino groups of each which are within bridging distance by DMA to the other (the number of which is expected to be small) would become either monofunctionally modified or coupled in an intra-chain fashion. The large amount of complex which is experimentally observed would not be formed.

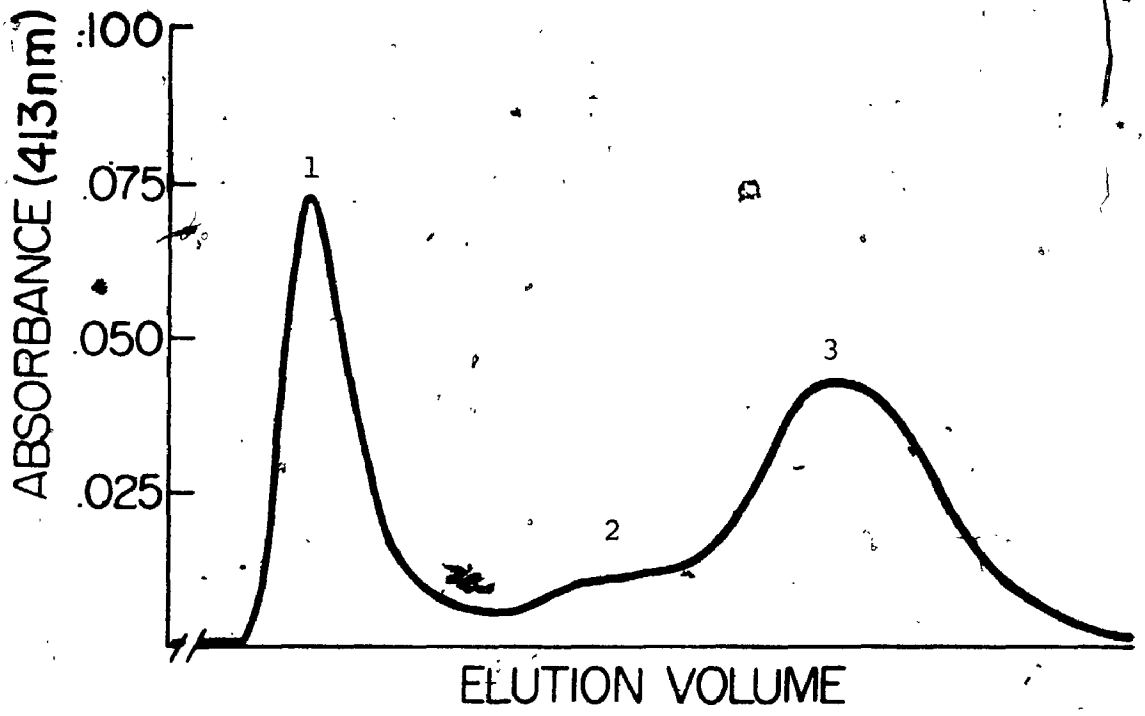
When (Hp + α^{SH}) was reacted with DMA in 2 M NaCl, complex formation was so extensive that dimer presence was virtually undetectable and that of monomer was slight (Figure 64). Elevated ionic strength was shown earlier to make more positive the change in free energy involved in α dimer dissociation, or in other words, to reduce the tendency of α dimer to dissociate. This in turn has made dimer dissociation from the surface of haptoglobin, as evidenced by the amount of complex formed, much less pronounced. This observation of seemingly slight significance could be lost in the sea of words that is this thesis but it can also be the keynote of some interesting ideas.

Figure 63: Sephadex chromatography of a solution of α^{SH} and haptoglobin following reaction with dimethyl adipimidate. The hemoglobin binding capacity method was used with G-75 rather than G-100 gel and PO_4-EDTA as buffer.

Assigned component identities:

1. DMA-crosslinked haptoglobin-α^{SH} complex
2. DMA-crosslinked α^{SH} dimer
3. DMA-reacted α^{SH} monomer

Figure 64: Sephadex chromatography of a solution of α^{SH} and haptoglobin following reaction with dimethyl adipimidate in the presence of 2 molar NaCl. The method and assigned component identities are as in Figure 63.


ABSORBANCE (413nm)
.100
.075
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1
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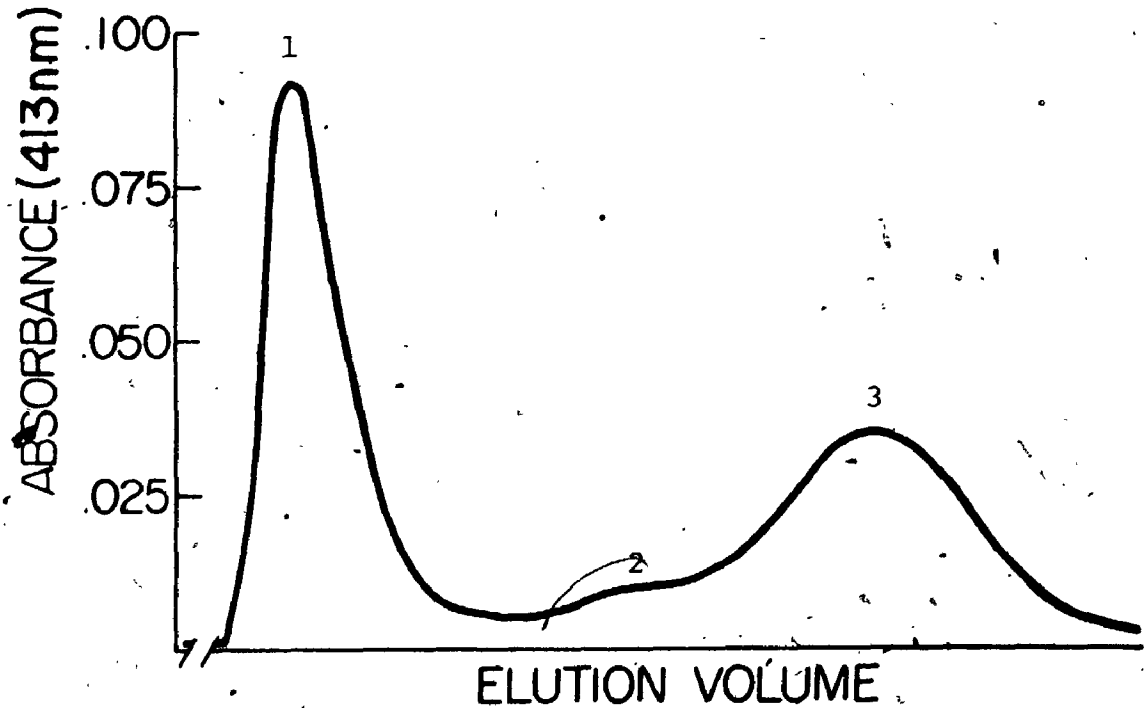
ELUTION VOLUME


ABSORBANCE (413nm)
.100
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ELUTION VOLUME

Discussion of the Binding of Hb and its Subunits to Hp

There is here a perplexing situation. Two facts have been established which must be integrated:

1. Alpha subunits of hemoglobin bind only weakly to haptoglobin such that the complex formed can be studied only in an α-containing environment. Attempts to isolate the complex without first chemically stabilizing it with, for example, imidoester bridges, fail as a result of rapid dissociation to free α^{SH} and Hp.

2. A dimer α^{SH}, artificially maintained, forms a complex with Hp which approaches the stability of HpHb. It survives passage through a 0.9 x 25 cm column of G-75 Sephadex, each bead of which can trap the dimer upon dissociation from Hp, and thus retard its migration with respect to Hp causing it to elute later than gel-excluded complex. Slight trailing is observed (Figure 62) but the fact remains, this complex possesses considerable stability.

A molecule of haptoglobin complexes stoichiometrically with one molecule of hemoglobin, $(\alpha\beta)_2$. Experimental results presented in this thesis indicate, in harmony with the work of Nagel and Gibson (1971), that two α subunits, not four, bind to Hp even in the presence of a high α:Hp molar ratio. Nagel and Gibson (1971) have questioned the very existence of β-specific sites prior to the attachment of α to Hp on the basis of the apparent lack of affinity between Hp and isolated β subunits. Thus

haptoglobin has two binding sites for α subunits as well as two existing or induced β sites. All of these are subunit-specific despite the known primary and tertiary structural similarities of the subunits (Dayhoff, 1969; Perutz, 1965).

The imidoester crosslinked α dimer could thus be thought to interact with various combinations of these α and β sites or even with one of more non-α non-β sites to which complementarity has been created by the chemical modification. It is unlikely that more than two areas of attachment are involved. Therefore the following possibilities can be considered:

Binding to:
1. a single α site
2. a single β site
3. a single non-α non-β site X
4. a single non-α non-β site Y
5. two non-α non-β sites XX or XY
6. a single α or β site and a non-α non-β site X or Y
7. two α sites
8. two β sites
9. an α and a β site

Although the paucity of concrete molecular-level knowledge concerning this system will not allow the elimination of any of the above, let us discuss their relative plausibility.

Protein modification by an imidoester such as dimethyl adipimidate entails certain well defined chemical changes. The pK of reacted amino groups is raised slightly from 9.6 to 10.6 (Hunter and Ludwig, 1962). Near pH neutrality, the degree of ionization of these groups closely ressembles that of the unmodified form. Thus the surface charge of DMA-α in the ionic conditions employed to investigate interaction with haptoglobin is also virtually unchanged. DMA hydrocarbon backbones are introduced but are similar to the side chain of lysyl residues prevalent on the α surface (Perutz, 1969). This implies that the chemical nature of the subunit does not radically change upon DMA modification. However, are conformational changes likely?

Imidoester crosslinking is not known to disrupt protein structure and in fact may confer some resistance to denaturation by maintaining the proximity of surface amino groups as they existed prior to reaction. As described earlier, the stability of the hemoglobin α subunit was carefully investigated while exposed to the crosslinking conditions and no signs of disruption were detectable spectrally or by gel-exclusion chromatography. Thus we can assume that the conformation present in the DMA α-dimer is essentially that which exists in the dimer formed by native α subunits. It follows therefore that the topography of the α subunit remains essentially unchanged.

Hence it is highly unlikely that the subunit acquires an affinity for a hypothetical non-α non-β site X or Y. In keeping with this assessment, ethyl acetimidate-reacted α subunits which probably closely ressemble DMA-α subunits, were not found to possess enhanced affinity for haptoglobin.

The particular surface of the α and β subunits by which they bind to haptoglobin have not been elucidated. However those surfaces which are internal when existing in the tetramer and form the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ interfaces are highly complementary and diverse in nature. The remaining surface area, external to the tetrameric subunits, is more uniform and studded with essentially all of the amino groups (Perutz, 1969). It is the latter which react with imidoester reagents thereby changing only slightly and in the direction of becoming more uniform. Thus whatever the surface of the α or β subunits which the haptoglobin α and β -specific sites are designed to accept, imidoester modification should not confer complementarity.

In principle, binding to two sites rather than one would be expected to provide greater interactive strength. However since the single interactions considered appear to be non-existent or necessarily slight, the dual-site possibilities are also disfavoured. Those involving the hypothetical sites X or Y would be particularly unlikely as they would require very fortuitous spatial

arrangement of X and Y, or X or Y and an α or β site.

Thus all the possibilities proposed above have been duly considered and seem improbable. No insight has been gained as to why DMA-α dimer binds strongly to haptoglobin while DMA-α monomer and ethyl acetimidate α subunits do not possess a similar capability. The approach to the nine possibilities however has involved one underlying assumption: that the affinity of α dimer for haptoglobin is in some way a result of imidoester crosslinking per se. Perhaps the truth lies elsewhere.

There is a way out of this impasse and that is to consider that the introduction of imidoester crosslinks does not induce conformational change in the hemoglobin α subunit but rather maintains one that occurs when single monomers combine to form dimers.

What information is available that might relate to this hypothesis? Unfortunately no direct evidence has been gathered to date. To this end, circular dichroism studies at various α subunit concentrations would seem to be a productive avenue of investigation. However indirect support can be obtained by consideration of a system of more general interest, that of the interaction of α with β subunits and in turn their interaction with haptoglobin.

The three-dimensional structures of the isolated subunits have not been determined X-ray crystallographically

so detailed comparison with their form in the tetramer is not yet possible.

Some information bearing on this point is available, however. No change in visible spectrum occurs upon separation of the hemoglobin α and β subunits (Antonini, Bucci et al, 1965) which implies that tertiary structure in the heme region is unaffected. Nagai, Sugita and Yoshimasa (1969) reported considerable differences in the circular dichroism of reconstituted hemoglobin and the sum of its isolated subunits and stated that this "reflects conformational changes of α and β chains when they are integrated into native hemoglobin". Since hemoglobin is a loosely held dimer of tightly bound dimers, this alteration probably occurs upon the association of single α and β monomers to form dimer and does not require forces present in the tetramer. α monomers also associate to form dimers and as discussed earlier it is reasonable to suggest that they do so in an attempt to remove from an aqueous environment that essentially non-polar surface involved in the hemoglobin $\alpha_1\beta_1$ interfate. Thus it is conceivable that to increase complementarity and in turn solvent exclusion, a conformational change occurs as two α subunits associate which is analogous to that known to take place upon the mixing of α and β subunits.

How does this relate to interaction with haptoglobin? First let us lay a groundwork to this subject.

Involvement of both hemoglobin subunits in formation of complex is illustrated by the extensive fluorescence quenching upon their sequential addition to haptoglobin (Nagel and Gibson, 1967). In particular, the role of β is not unlike the central interlocking piece in a jigsaw as upon its binding to the Hpα complex there is a tremendous rise in stability.

Yet isolated β does not bind to free haptoglobin (Chiancone, Alfsen et al, 1968). This caused Nagel and Gibson (1971) to suggest the induction of a β site on Hp upon binding of α. A second possibility not previously proposed is that a conformational change in the β subunit is induced by its association with α as a dimer. This would make it complementary to a β site present on the haptoglobin surface independent of the binding of α. In fact dimer formation may bring about changes in both isolated subunits thus increasing α complementarity to Hp as well. This last point is attractive as it is difficult to convincingly reconcile the weak binding of isolated α subunits to Hp with the tremendous stability of HpHb complex (K_{Assoc} of the order of 10^9: Clark, 1966) without invoking some mechanism of strengthening the Hp-α interaction. It is also conceivable that Hp undergo some tertiary modification as the complex is formed.

The binding of isolated α and β subunits to Hp is demonstrably weak. Evidently the driving force of subunit

transition from an isolated to a form more suitable for binding to Hp is incorporation into an αβ dimer. Otherwise isolated subunits would undergo this change and bind strongly to Hp. The transition may involve further projection of non-polar areas towards the subunit exteriors which would be highly unfavourable if not insulated from the polar aqueous environment by coverage with a similar surface. The slight interaction of isolated α with Hp when compared with the apparent lack of β-Hp interaction would imply that structural modification of β is less extensive than that of α or that β self-interaction as β_4 is much more favourable than as monomer or dimer with Hp.

The hypothesis that hemoglobin α and β subunits induce in each other conformational changes by which they acquire a greatly enhanced affinity for haptoglobin is consistent with our present knowledge of these subunits and of protein behavior generally. Returning to the case of the α dimer, it is evident that an analogous tertiary change, maintained by DMA crosslinks, could explain the differences in affinity for Hp of DMA-α_2, DMA-α and ethyl acetimidate α. It is solely DMA-α_2 in which monomer-induced structural rearrangement can occur and as is seen experimentally, it is DMA-α_2 only that exhibits significant interaction with haptoglobin.

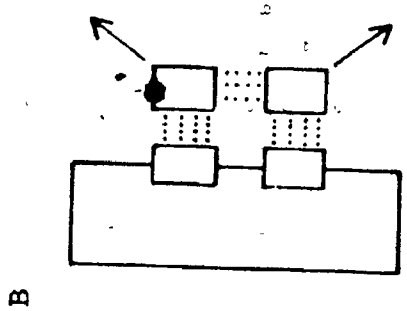
By gel filtration, sedimentation equilibrium and velocity it has been shown that α forms dimer reversibly

at concentrations similar to those used to study binding to haptoglobin. However the absence of complex isolated upon gel chromatography of Hp and α subunits indicate that $Hp\alpha_2$ complexes are not sufficiently stable to dissociation during the time required for passage through the column. It was only by maintaining a constant α environment as in the supra plateau and sedimentation velocity experiments that Hpα subunit complex was observed. It has also been shown by gel filtration that the DMA-α_2 Hp complex is significantly resistant to breakdown. These facts are consistent in view of a basic stability principle of multi-association products. There is a distinct advantage in binding two entities as a common unit to a third rather than separately. Figure 65 diagrammatically illustrates this point using the $Hp\alpha_2$ system as an example. When the two subunits are covalently linked, the complex is disrupted only upon simultaneous breakage of the two Hp-interactions. The freedom of two non-covalently linked subunits to dissociate from the haptoglobin surface is indicated by arrows.

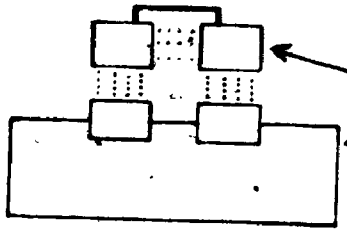
Figure 65: A schematic diagram illustrating:

A. The binding of α dimer as a crosslinked single unit to haptoglobin.

B. The binding of α dimer not maintained by an imidoester crosslink. The freedom of the two non-covalently linked subunits to dissociate from the haptoglobin surface is indicated by arrows.



B



A

DMA-α_2 Dimer

Haptoglobin

The forces involved in α dimer formation, in the absence of high ionic strength, are very weak as indicated by the calculated ΔG_d^o. Due to thermal vibration the subunits most certainly do not rest together on the Hp molecule as static masses but rather move with respect to one another. As their closeness and interaction decreases to some unknown critical point, the induced conformation is lost as is enhanced complementarity for haptoglobin. Dissociation therefore ensues.

In contrast, the αβ dimer possesses much greater stability as is experimentally illustrated by the failure to dissociate detectably to monomers at concentrations as low as 10^{-8} molar (Bucci, 1971; Kellet and Schachman, 1971). This dimer therefore bonds to haptoglobin essentially as a single entity thereby necessitating breakage of both Hp-α and Hp-β interactions to allow complex dissolution.

Based on the absence of a trailing edge of αβ dimer upon gel exclusion chromatography, the Hp αβ complex is clearly more stable than Hp-DMA α_2 complex. This could be due to greater αβ dimer complementarity for the Hp surface and/or the fact that α_2, when crosslinked by the 8.6 Å long DMA reagent, still has sufficient spatial freedom at maximum DMA extension to allow reversion to isolated monomer conformation and hence dissociation from Hp. The use of a bifunctional imidoester such as malonimidate (maximum span of 4.8 Å) may result in a cross-

linked α_2 dimer which may interact with haptoglobin with equivalent affinity but greater stability. (Reagent lengths were a personal communication from D. B. Smith.)

The induced fit hypothesis of dimer formation and resulting enhanced interaction with haptoglobin presented at some length above is supported by the strong interaction of DMA-α_2 with Hp and is consistent with the literature. A further test however can be described.

The β^{SH} subunit of Hemoglobin exists as a tetramer which is thought to closely ressemble hemoglobin A. Therefore crosslinking with a bifunctional imidoester will bridge only lysyl residues which are exterior in β_4 and $(\alpha\beta)_2$ (Perutz, 1965, has indicated their total external distribution in the Hb tetramer). It is proposed that crosslinking two amino groups which are proximal in the isolated β_4 state but which become distant in $\alpha\beta$ dimer be attempted. Such an imidoester bridge would therefore prevent the structural transition. Addition of α^{SH} would result in the very favourable $\alpha_1\beta_1$ dimer where possible. If some of the β material appears after gel filtration to be monomer or tetramer dissociable to monomer in SDS-PAGE, this would indicate an inability to form $\alpha\beta$ dimer due solely to a covalent constraint far from the surface which normally interacts with α. This would support the spectral evidence of Nagai, Sugita and Yoshimasa (1969) that hemoglobin subunits experience

conformational changes as they become integrated into a tetramer and provide chemical proof of obligatory β structural transition when forming αβ dimer. In addition an assay of the ability of this β material to form complex with Hp + α^{SH} would be predicted as negative.

This experimental approach could also be applied to the α as opposed to the β subunit. The reaction would best be performed in a concentration range chosen to assure a minimal dimer presence so as to avoid any non-isolated-α subunit conformational states. The resulting intramonomer crosslinked α material could then be investigated by gel filtration to detect the existence of a fraction unable to form αβ dimer with added β and to form complex with added Hp + β. (Most of the material would be expected to interact normally with β and Hp + β since it would possess no crosslinks which inhibit the proposed structural transition). That fraction unable to dimerize could then be studied by the sedimentation velocity and sedimentation equilibrium techniques to ascertain its ability to form α dimer. Should such a fraction exist and it be found incapable of forming α_2 and αβ dimer, obligatory structural transition of the α subunit would be indicated. Should this material also be incapable of forming complex in the presence of β and Hp, then a similar conclusion could be made regarding the strong binding of α subunit to haptoglobin seen in HpHb.

Let us now reconsider the particular areas of the haptoglobin surface to which the α_2 dimer binds. As discussed earlier, two distinct sites are probably involved and it is unlikely that they be non-α non-β in nature. By elimination, there remain the cases of two α-specific sites or an α-plus β-specific site. If the latter is true, then haptoglobin should be able to bind two DMA-α_2 dimers on the basis of two facts. Haptoglobin has been shown to accept two αβ dimers independently and with comparable affinity (Nagel and Gibson, 1971). Also, the size of the α monomer is virtually identical to that of β. Since this size is essentially unchanged by DMA modification, spatial crowding should not therefore prevent the binding of two DMA-α_2 dimers. Thus an experiment can be designed to differentiate between the two possibilities simply by determining whether Hp becomes saturated by one or by two DMA-α_2 dimers.

A procedure such as that which resulted in the profile presented in Figure 61 involving gel filtration separation of Hp-DMA α_2 complex from unbound subunits is not adequate since the loss of dimers during the separation process, as indicated by the trailing edge of the complex peak, would result in a low estimate of saturation. Thus it is apparent that the complex must be stabilized by crosslinking to prevent this dissociation or studied in a [redacted]-α_2 environment.

A heterogeneous mixture of $Hp\alpha_n$, n = 0 →4, could result from intramolecularly crosslinking Hp in the presence of α monomer. Gel exclusion chromatographic purification of these components would be difficult due to the small molecular weight increment contributed by each α monomer. In view of these observations, the following experiment is proposed.

Gel filtration of ultrafiltered, millipore filtered, DMA crosslinked α results in two major fractions, DMA-α and DMA-α_2, as well as a small amount of high molecular weight aggregate (see Figure 61). Sedimentation velocity plateau height analysis such as was used to discover the number of isolated unmodified α monomers which bind to haptoglobin can then be applied to a sample of Hp + DMA-α_2 to indicate the number of α monomers bound by Hp as DMA-α_2 dimers. It can be predicted that haptoglobin accept two DMA-α_2 dimers in view of their similarity to $\alpha_1\beta_1$ as discussed earlier and the fact that two subunits in HpHb complex are within bridging distance by DMA (Lockhart and Smith, in press). It seems unlikely that the two α sites be sufficiently close as well considering the independent binding of hemoglobin αβ dimers. It follows that if the DMA-α_2 dimer interacts with an α and a β site on Hp, then two such dimers must bind as Hp possesses two of each type of site.

A schematic diagram illustrating the proposed

conformational changes in the α and β subunit upon their association as an αβ dimer and the resulting increase in affinity for Hp is presented in Figure 66. Figure 67 indicates how this concept has been applied to the case of the α subunit forming a dimer which when maintained by imidoester crosslinks forms a relatively stable complex with haptoglobin. In the latter representation, interaction of the α dimer with an α and β site of Hp is assumed.

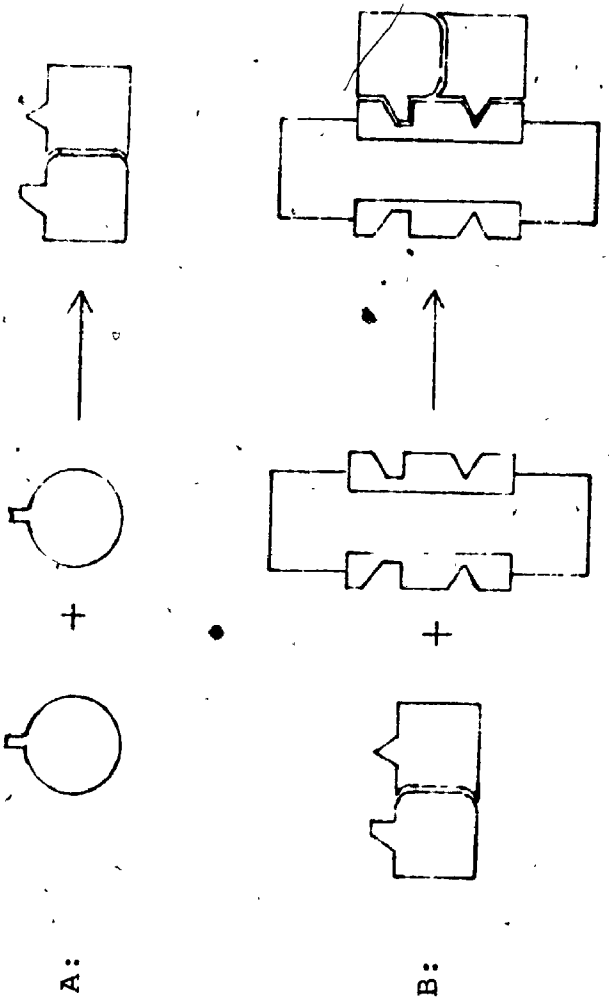
An alternate and promising method of forming an α_2 dimer with an $\alpha_1\beta_1$ orientation is to react α with one of the bivalent mercurials described by Straessle (1951). Since the subunit possesses only one sulfhydryl (Dayhoff, 1969) which lies very near the $\alpha_1\beta_1$ contact surface (Perutz, 1965), any covalently linked dimer would be expected to have this structure. An ability of this dimer to form complex with Hp would further extend the work presented here with DMA-α_2 as it would virtually eliminate the $\alpha_1\beta_1$ interface as that involved in HpHb. In addition, it would prove that α existing as a dimer analagous to $\alpha_1\beta_1$ of hemoglobin is a form which interacts with Hp thus lending support to the above induced fit interpretation of hemoglobin subunit interactions and the hypothesis related to their affinity for haptoglobin.

A longstanding approach to the topographical elucidation of hemoglobin binding to haptoglobin consists of narrowing the number of possible surfaces involved

Figure 66: A schematic diagram illustrating:

A. The proposed conformational changes in the α and β subunit upon their association as an αβ dimer.

B. The resulting enhanced affinity for Hp.


+
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A:
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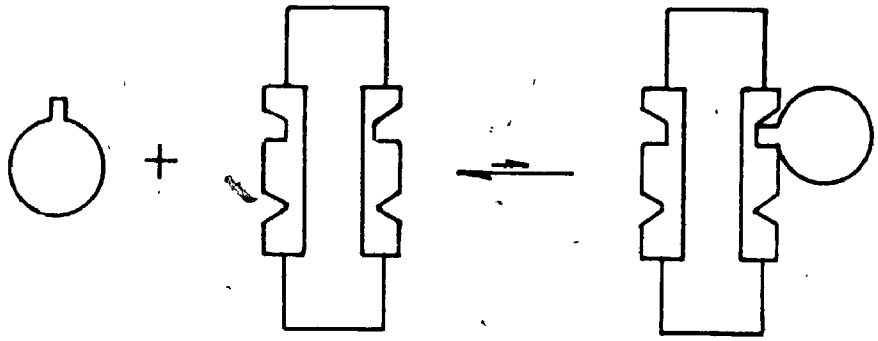
Figure 67: A schematic diagram illustrating an induced fit hypothesis designed to reconcile the weak interaction of isolated α monomers and Hp with the relatively strong binding of DMA-cross-linked α dimer to haptoglobin.

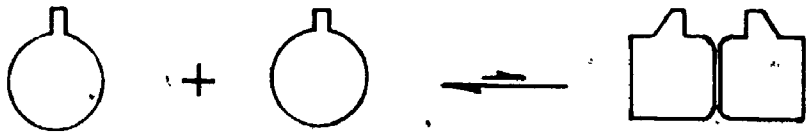
A. Formation of Hpα complex (Half-saturation)

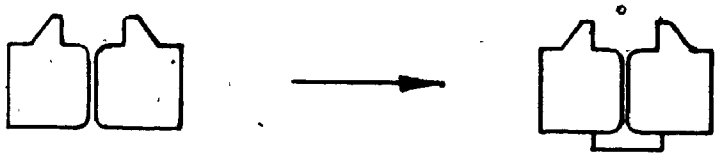
B. Formation of α_2 dimer

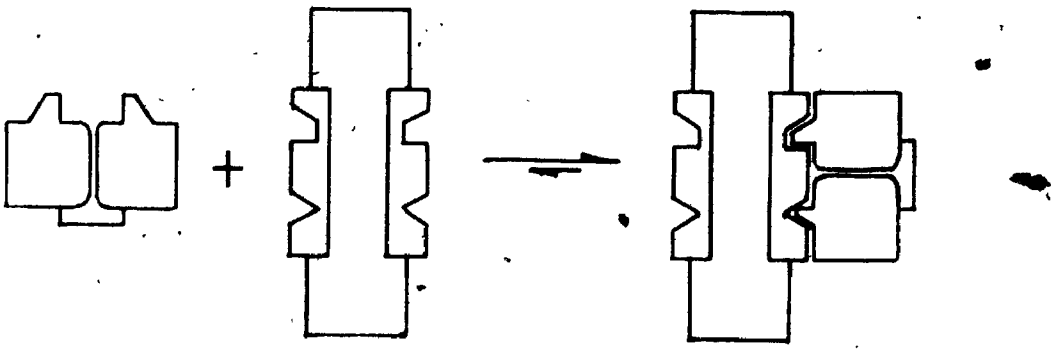
C. Formation of DMA-α dimer

D. Formation of Hp-DMA α_2 complex (Half-saturation).









such that by a process of elimination that one which participates can be determined. The work presented in this thesis suggests that to the growing list can be added the hemoglobin $\alpha_1\beta_1$ contact surface. In view of evidence reported by other authors and the known structure of hemoglobin dimer, the $\alpha_1\beta_2$ contact area is strongly implicated.

SUMMARY

The preparation of porcine haptoglobin in gram quantities using techniques previously reported in isolation procedures was found to be complicated by the spontaneous generation of dimer albumin. Preparative gel electrophoresis yielded uncontaminated Hp but was limited to milligram quantities. Affinity chromatography with Concanavalin A-Sepharose 4B provided a rapid method of purifying Hp to homogeneity from a sample which had been rendered free of other glycoproteins by gel filtration. Sample size was restricted only by volume of gel employed.

Microheterogeneity of porcine haptoglobin was noted with at least 14 components separable by PAGE. All of these microforms were capable of binding hemoglobin and were thought to result from gradual and perhaps enzymatically catalyzed loss of carbohydrate moieties. Treatment with neuraminidase converted them into a product of single electrophoretic mobility.

The α^{SH} and α^{PMB} subunits of hemoglobin were

prepared by the method of Geraci et al (1969) with modification. Subunit protection against oxidation by metallic ions was afforded by the inclusion of 2×10^{-5} M EDTA in all solutions employed in their preparation and use. Conditions of 0.1 M phosphate pH 7.5 proved conducive to subunit stability and were chosen for storage and extensively used for experiment. In addition, extension of the method by G-75 gel filtration was found to be necessary to remove a contaminant found to be Hb. Sulfhydryl regeneration of the α^{SH} subunits was complete and they were electrophoretically pure. In contrast, α^{PMB} exhibited two bands of unequal proportions in PAGE. An alternate protocol of greater simplicity and more suited to higher concentration and yield was proposed.

As a preliminary to Hp:α interaction studies, the extinction value of α^{SH} was determined at 368 nm as was its stability to acidic and basic conditions.

Attempts made to isolate complex formed by Hp and α^{SH} were fruitless and considered impractical. Plateau height analysis of (Hp + α) sedimentation behavior indicated the presence of an equilibrium with maximum binding of $2\alpha^{SH}$ per Hp at a 6 fold α:Hp molar ratio. This was supported by comparison of various sedimentation coefficients and confirmed by supra plateau gel filtration studies. This has resolved the debate in the literature as to the stoichiometry of Hpα complex formation.

A self-association equilibrium of α^{SH} was indicated by the plateau edge method of Winzor and Scheraga (1963) and by sedimentation equilibrium ultracentrifugation. This phenomenon was also shown but to a lesser extent by α^{PMB}. Association significantly increased with ionic strength. A dissociation constant and ΔG_d^o was calculated for α^{SH} reversibly forming dimers in the absence and presence of 2 M NaCl. It was proposed that the α_2 entity was analogous to the hemoglobin $\alpha_1\beta_1$ dimer.

Crosslinking α^{SH} with dimethyl adipimidate in conditions which yield intramolecular bridges resulted in two DMA-α dimers as seen in SDS-PAGE. DMA-α_2 was found to form a significantly stable complex relative to DMA-α monomer, ethyl acetimidate-modified α and unreacted α. α^{SH} reacted with DMA in the presence of Hp yielded more complex than when the Hp was added subsequent to crosslinking. This was particularly true at elevated ionic strength.

The following theory was proposed: α and β subunits undergo a conformational transition upon association as an $\alpha_1\beta_1$ dimer which makes them complementary to Hp. α subunits spontaneously form a dimer analogous to $\alpha_1\beta_1$ and experience a similar transition which confers complementarity to Hp. When this dimer is artificially maintained by DMA, significant binding affinity for Hp results. It

was also suggested that interaction with DMA-α_2 involves an α- and β-specific site on the Hp surface and that both exist in free Hp. Two DMA-α_2 dimers are therefore predicted to bind to haptoglobin.

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